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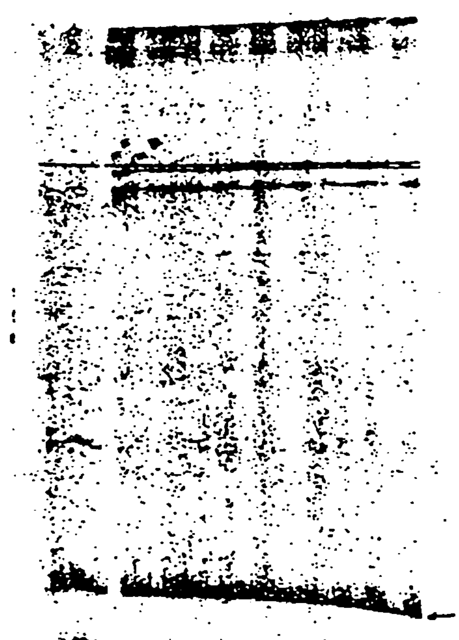

(57) Abstract

The present invention relates to the identification of the autosomal dominant polycystic kidney disease (PKD) gene and high throughput assays to identify compounds that interfere with PKD activity. Interfering compounds that inhibit the expression, synthesis and/or bioactivity of the PKD gene product can be used therapeutically to treat polycystic kidney disease.

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IDENTIFICATION OF POLYCYSTIC KIDNEY DISEASE GENE, DIAGNOSTICS AND TREATMENT

This is a continuation-in-part of U.S. Serial No. 08/253,524, filed, June 3, 1994, which is incorporated by reference herein in its entirety.

1. INTRODUCTION

The present invention relates to the identification of the gene, referred to as the PKD1 gene, mutations in which are responsible for the vast majority of cases involving autosomal dominant polycystic kidney disease (ADPKD). The PKD1 gene, including the complete nucleotide sequence of the gene's coding region are presented. Further, the complete PKD1 gene product amino acid sequence and protein structure and antibodies directed against the PKD1 gene product are also presented. Additionally, the present invention relates to therapeutic methods and compositions for the treatment of ADPKD symptoms. Methods are also presented for the identification of compounds that modulate the level of expression of the PKD1 gene or the activity of mutant PKD1 gene product, and the evaluation and use of such compounds in the treatment of ADPKD symptoms. Still further, the present invention relates to prognostic and diagnostic, including prenatal, methods and compositions for the detection of mutant PKD1 alleles and/or abnormal levels of PKD1 gene product or gene product activity.

2. BACKGROUND OF THE INVENTION

Autosomal dominant polycystic kidney disease (ADPKD) is among the most prevalent dominant human disorders, affecting between 1 in 1,000 and 1 in 3,000 individuals worldwide (Dalgaard, O.Z., 1957, Acta. Med. Scand. 158:1-251). The major manifestation of the disorder is the progressive cystic dilation of renal tubules (Gabow, P.A., 1990, Am. J. Kidney Dis. 16:403-413), leading to renal failure in half of affected individuals by age 50.

ADPKD-associated renal cysts may enlarge to contain several liters of fluid and the kidneys usually enlarge progressively causing pain. Other abnormalities such as pain, hematuria, renal and urinary infection, renal tumors, salt and water imbalance and hypertension frequently result from the renal defect. Cystic abnormalities in other organs, including the liver, pancreas, spleen and ovaries are commonly found in ADPKD. Massive liver enlargement occasionally causes portal hypertension and hepatic failure. Cardiac valve abnormalities and an increased frequency of subarachnoid and other intracranial hemorrhage have also been observed in ADPKD. Progressive renal failure causes death in many ADPKD patients and dialysis and transplantation are frequently required to maintain life in these patients. Although end-stage renal failure usually supervenes in middle age (ADPKD is sometimes called adult polycystic kidney disease), children may occasionally have severe renal cystic disease.

Although studies of kidneys from ADPKD patients have demonstrated a number of different biochemical, structural and physiological abnormalities, the disorder's underlying causative biochemical defect remains unknown. Biochemical abnormalities which have been observed have involved protein-sorting, the distribution of cell membrane markers within renal epithelial cells, extracellular matrix, ion transport, epithelial cell turnover, and epithelial cell proliferation. The most carefully documented of these findings are abnormalities in the composition of tubular epithelial cells, and a reversal of the normal polarized distribution of cell membrane proteins, such as the Na^+/K^+ ATPase (Carone, F.A. et al., 1994, Lab. Inv. 70:437-448.).

As the name implies, ADPKD is inherited as an autosomal dominant disorder. Three distinct loci have been shown to cause phenotypically indistinct forms of the disease, with greater than 85-90% of disease incidence being due to mutations which map to the short arm of chromosome 16, as

discussed below. Despite intensive investigation, the molecular defect responsible for ADPKD is not known.

In 1985 Reeders et al. (Reeders et al., Nature 317:542, 1985) carried out genetic linkage studies of a large number of ADPKD families and demonstrated that a gene on the short arm of chromosome 16 was mutated in most cases of ADPKD. This gene has been designated PKD1 by the Nomenclature Committee of the Human Gene Mapping Workshop and the Genome Data Base of the Welch library, John Hopkins University. Further linkage studies have identified a set of genetic markers that flank the gene-rich region containing the PKD1 gene (Reeders et al., 1988, Genomics 3:150; Somlo et al., 1992, Genomics 13:152; Breuning et al., 1990, J. Med. Genet. 27:603; Germino et al., 1990, Am. J. Hum. Genet. 46:925). These markers have been mapped by a variety of physical mapping techniques including fluorescent in situ hybridization and pulsed-field gel electrophoresis (Gillespie et al., 1990, Nucleic Acids Research 18:7071). It has been shown that the closest distal genetic marker (D16S259; on the telomeric side of the PKD1 locus) lies within 750 kb of the closest proximal genetic marker (D16S25; on the centromeric side of the PKD1 locus). The interval between the genetic markers has been cloned in a series of overlapping cosmid and bacteriophage genomic clones (Germino et al., 1992, Genomics 13:144), which contain the entire PKD1 interval, with the exception of two gaps of less than 10 kb and less than 50 kb. Restriction mapping of these clones has confirmed that the interval between the flanking genetic markers is 750 kb.

While genetic mapping studies such as these have begun to narrow the region within the human genome in which the gene responsible for ADPKD lies, there exist an estimated twenty or more genes within this 750 kb interval. Given the prevalence and severity of ADPKD, however, it is of great importance to eludicate which, if any, of these postulated genes corresponds to PKD1.

3. SUMMARY OF THE INVENTION

The present invention relates to methods and compositions for the diagnosis and treatment of autosomal dominant polycystic kidney disease (ADPKD). Specifically, a novel gene, referred to as the PKD1 gene, is described in Section 5.1. Mutations within the PKD1 gene are responsible for approximately 90% cases of ADPKD. Additionally, the PKD1 gene product, including the nucleotide sequence of the complete coding region is described in Section 5.2. Antibodies directed against the PKD1 gene product are described in Section 5.3.

Further, the present invention relates to therapeutic methods and compositions for the amelioration of ADPKD symptoms. These therapeutic techniques are described in Sections 5.9 and 5.10. Methods are additionally presented for the identification of compounds that modulate the level of expression of the PKD1 gene or the activity of PKD1 mutant gene products, and the evaluation and use of such compounds as therapeutic ADPKD treatments. Such methods are described in Section 5.8.

Still further, the present invention relates to prognostic and diagnostic, including prenatal, methods and compositions whereby the PKD1 gene and/or gene product can be used to identify individuals carrying mutant PKD1 alleles, exhibiting an abnormal level of PKD1 gene product or gene product activity. Additionally, the present invention describes methods which diagnose subjects exhibiting ADPKD symptoms. Such techniques are described in Section 5.12. Additionally, the present invention relating to the use of PKD1 animal knockout screening assays for the identification of compounds useful for the amelioration of ADPKD symptoms.

The coding region of the PKD1 gene is complex and extensive, having a size of approximately 60 kb and containing a total of 46 exons, the sequence of which, until now, has been difficult to obtain for a number of reasons. First, the majority (approximately the first two thirds) of the PKD1 gene is duplicated several times in a transcribed

fashion elsewhere in the genome, thus making it very difficult to distinguish authentic PKD1 sequence from PKD1-like sequence. Further, the PKD1 gene contains extensive repeated regions of high GC content which are not only difficult to sequence accurately, but, additionally, make the alignment of PKD1 nucleotide sequence extremely difficult. Still further, the PKD1 gene encodes a large transcript of approximately 14.5 kb in length, and evidence exists that there are alternatively spliced forms of the gene. Thus, the size of the PKD1 gene, the size and complexity of PKD1 transcript, coupled with the above-described PKD1 features made the successful sequencing of the gene and its cDNA very difficult. As described in Sections 5.1.2 and in the Example presented in Section 10, below, however, the obstacles to sequencing the PKD1 gene have now, for the first time, been overcome.

The PKD1 transcript, which is approximately 14.5 kb in length, encodes a PKD1 gene product with a derived amino acid sequence of 4304 amino acid residues. This PKD1 gene product contains at least five distinct peptide domains which are likely to be involved in protein-protein and/or protein carbohydrate interactions. Further, this PKD1 gene product shares amino acid sequence similarity with a number of extracellular matrix proteins. These features of the PKD1 gene product indicate that ADPKD is caused by a biochemical defect involving extracellular signalling and/or extracellular matrix assembly, and suggests therapeutic strategies whereby ADPKD can be treated and/or whereby ADPKD symptoms can be ameliorated.

The Examples described in Section 6 through 11, below, demonstrate the successful identification and characterization of the PKD1 gene and gene product, including the complete nucleotide sequence of the PKD1 coding region, the complete amino acid sequence, and the elucidation of the protein structure of the PKD1 gene product. Further, a ADPKD-causing mutation is identified and described.

4. DESCRIPTION OF THE FIGURES

FIG. 1. A map of the PKD1 interval showing the cosmids and bacteriophage clones covering the region (Taken from Germino et al, 1992, Genomics 13:144.) The PKD1 region as defined by flanking markers extends from D16S259 (pGGG1) to D16S25, a span of approximately 750kb. Single-copy probes used in pulsed-field gel mapping of the region are shown above the line (pGGG1, CMM65b, etc.). C, M, P, N and B are sites for restriction enzymes *Cla*I, *Mlu*I, *Pvu*I, *Not*I and *Bss*HII, respectively. Sites that cleave in genomic DNA from only some tissues are shown in parenthesis. Bold bars (a-z, aa) represent the extents of the coding regions (see Table 2). Horizontal lines 1-38 represent cosmid and phage clones spanning the PKD1 region, as shown here:

1=cJC1	9=cDEB11	17=cKLH4	25=cNK30
2=cJC2	10=cGGG10	18=cKLH6	26=λLCN1w1
3=cDEB1	11=cGGG1	19=cKLH7	27=λLCNw2J2
4=CDEB4	12=cGGG2	20=cKLH8	28=λLCNw1w3
5-cDEB7	13-cGGG3	21=cKLH9	29=λLCNw5.2
6=cDEB8	14-cGGG4a	22-cNK32	30=λNK92.6w5.1
7=cDEB9	15=cGGG4b	23=cNK31	31=λNK92.6w4.1
8=cDEB10	16=cGGG6	24=cGGG8	32=cNK92.6w1.3

33=cNK92.6
34=cNK92.2
35=cNK63.7
36=cNK14
37=cCOS4
38=cCOS3

FIG. 2. A map of the PKD1 region as defined by flanking markers. The region extends from D16S259 (pGGG1) to w5.2CA, a microsatellite repeat that lies within λLCNw5.2, a span of approximately 480kb. The labels are as for FIG. 1.

FIG. 3A-B. Genomic DNA from 40 unrelated ADPKD patients was amplified by PCR for SSCP analysis. Primers F23 and R23 (See Table 1, below) were used to amplify an exon of 298bp. Variant SSCP patterns were seen in two ADPKD patients under the following conditions. Each of the patients was heterozygous for the normal pattern and the variant pattern.

The pattern seen in these patients was not seen in normal individuals. Arrow indicates non-denatured DNA.

FIG. 4. A map (not to scale), derived from the cosmid contig cGGG1, cGGG10 and cDEB11, of the genomic region containing the PKD1 gene. The horizontal black bars show the positions of the three cosmids. The discontinuities in these bars indicate that the full extent of cGGG1 and cDEB11 are not shown. The map was constructed using restriction enzyme data from several enzymes. BamHI, EcoRI and NotI restriction sites are shown. The numbers below the horizontal line represent distances in kilobases between adjacent restriction sites. The PKD1 cDNA clones are shown above as grey bars. These clones hybridize to the restriction fragments shown immediately below them in the genomic map.

FIG. 5A. Structure of the PKD1 gene transcript. The bar at the top represents the PKD1 exon map. A total of 46 exons were identified. Below the gene transcript map are depictions of the overlapping cDNA clones, with putative alternatively spliced regions as indicated.

FIGS. 5B-5C. PKD1 exons. This chart lists PKD1 exon sizes and indicates which cDNA clones contain nucleotide sequences corresponding to sequences present within specific exons.

FIG. 6. PKD1 nucleotide and amino acid sequences. Depicted herein are, top line, the nucleotide sequence of the entire PKD1 coding region (SEQ ID NO: 1), and, bottom line, the PKD1 derived amino acid sequence (SEQ ID NO: 2), given in the one-letter amino acid code.

FIGS. 7A-7B. The derived amino acid sequence of PKD1 gene product (SEQ ID NO: 2). The putative peptide domains of the PKD1 gene product are depicted underneath the amino acid sequence.

FIG. 8. A schematic representation of the PKD1 gene product, with each of its putative domains illustrated.

FIG. 9. SSCP analysis. Genomic DNA from a total of 60 unrelated ADPKD patients was amplified by PCR for SSCP analysis. Intronic primers F25 and Mill-1R (see Section 10.1, below) were used for amplification. A variant SSCP pattern was seen in one individual. The amplified DNA from this individual was then reamplified with the intronic primers KG8-F31 and KG8-R35 (see Section 10.1, below). Both strands of the reamplied DNA were sequenced, using F25 and Mill-1R as sequencing primers. As discussed in Section 10.2, below, sequencing revealed a C to T transition which created a stop codon at PKD1 amino acid position 765. The pattern seen in these patients was not seen in normal individuals.

5. DETAILED DESCRIPTION OF THE INVENTION

Methods and compositions for the diagnosis and treatment of (ADPKD) are described herein. Specifically, the gene, referred to herein as the PKD1 gene, in which mutations occur that are responsible for the vast majority of ADPKD cases is described. Further, the PKD1 gene product and antibodies directed against the PKD1 gene product are also presented.

Therapeutic methods and compositions are described for the treatment and amelioration of ADPKD symptoms. Further, methods for the identification of compounds that modulate the level of expression of the PKD1 gene or the activity of mutant PKD1 gene product, and the evaluation and use of such compounds in the treatment of ADPKD symptoms are also provided.

Still further, prognostic and diagnostic methods are described for the detection of mutant PKD1 alleles, of abnormal levels of PKD1 gene product or of gene product activity.

5.1. THE PKD1 GENE

The PKD1 gene, mutations in which are responsible for greater than 9 in 10 cases of ADPKD, is described herein. Specifically, the strategy followed to identify the PKD1 gene is briefly discussed, as is the strategy for obtaining the complete nucleotide sequence of the gene. Further, the PKD1 nucleotide sequence and alternative splicing features are described. Still further, nucleic acid sequences that hybridize to the PKD1 gene and which may be utilized as therapeutic ADPKD treatments and/or as part of diagnostic methods are described. Additionally, methods for the production or isolation of such PKD1 nucleic acid molecules and PKD1-hybridizing molecules are described.

5.1.1. IDENTIFICATION OF THE PKD1 GENE

Prior to the present invention, it had only been known that the physical location of the PKD1 gene within the human genome was somewhere within a 750 kb chromosomal region on the short arm of chromosome 16. As presented herein, the interval in which this gene lies has now been reduced until the specific PKD1 gene has been identified out of this large portion of DNA.

Briefly, the strategy which was followed to identify the PKD1 gene is as described herein. First, as demonstrated in the Example presented in Section 6, below, the 750 kb PKD1 interval was first substantially narrowed to approximately 460 kb, via genetic linkage studies. Next, as shown in the Example presented in Section 7, below, a maximum of 27 transcriptional units (TUs) were identified within this approximately 460 kb PKD1 interval. The total length of these TUs was approximately 300 kb. Thus, the region containing the PKD1 coding region was narrowed down to a region of approximately 300 kb.

Next, as presented in the Example shown in Section 9, below, a Northern analysis was conducted with mRNA isolated from normal and ADPKD patient kidney tissue, in order to attempt to compare the pattern of ADPKD pathology to the

expression profile of the TUs within the PKD1 interval. One of the TUs, Nik9, was eliminated by such an analysis, which indicated undetectable expression in the kidney and liver.

In addition, as demonstrated in the Example presented in Section 9, below, a systematic search was undertaken using several independent techniques, including Southern analysis SSCP, DGGE and direct sequencing of coding sequences, to detect mutations in ADPKD patients within the TUs of the PKD1 region. By conducting such a mutation screen, greater than 80% of the combined identified coding sequences in the PKD1 region were excluded, thus further substantially narrowing down the region in which the PKD1 gene could lie. The screen was initially performed on individual genes until virtually all the coding sequences were shown to be devoid of mutations. The focus on possible PKD1 candidates was further honed by the recognition that PKD1 demonstrated one of the highest new mutation rates known for human diseases. Based on this observation, it was hypothesized that either the PKD1 gene contained a highly mutable site or that the gene presented a large number of potential mutation sites, each mutable at a regular frequency. Such a hypothesis is supported by the absence of substantial linkage disequilibrium among selected population groups. Further, this hypothesis predicted that if the PKD1 gene was a small transcript, it should contain a highly mutable element.

Trinucleotide repeat expansion represent one of the major sources for dominant mutations such as the ADPKD-causing mutations which arise in the PKD1 gene. A systematic search for such highly mutable trinucleotide repeats was conducted within the TUs in the remaining region wherein PKD1 could lie, but no such repeats were identified.

The only other explanation for the high mutational prevalence is that the gene is physically large and presents a large target for mutations. Of the TUs, nik823, within the potential PKD1 region that had not been excluded by other means, only two were of a size that could potentially support such a high mutation rate. As demonstrated in the Example

presented, below, in Section 9, a search for ADPKD correlative mutations within one of these TUs failed to identify any such mutations, causing it to be excluded as a candidate PKD1 gene. Ultimately, as demonstrated in the Example presented in Section 10, below, one of these polymorphisms has been shown to be a de novo mutation which is predicted to lead to the production of a truncated PKD1 protein in the affected individual, These finding are highly suggestive, if not proof, that the identified gene is the PKD1 gene.

Thus, the examples presented below in Sections 6 through 11 demonstrate, through a variety of techniques, the genetic and molecular characterization of the PKD1 region, and ultimately demonstrate that the PKD1 gene, dominant mutations in which cause ADPKD, has been identified.

5.1.2. SEQUENCING OF THE PKD1 GENE

As discussed, below, in Section 5.1.3, the nucleotide sequence of the entire coding region of the PKD1 gene has now successfully been isolated and sequenced. In order to achieve this goal, however, a number of PKD1-specific impediments had to be overcome. The strategy for obtaining the PKD1 gene sequence is discussed, briefly, in this Section. The Example presented below, in Section 11, discusses this sequencing strategy in more detail.

First, the PKD1 gene is very large, (approximately 60 kb), as is the PKD1 transcript, being approximately 14.5 kb in length. In addition to this size difficulty, approximately two thirds of the 5' end of the gene is duplicated several times in a highly similar, transcribed fashion elsewhere in the human genome (Germino, G.G. et al., 1992, Genomics 13:144-151; European Chromosome 16 Tuberous Sclerosis Consortium, 1993, Cell 75:1305-1315).

The near-identity of the sequence of cDNA derived from PKD1 and from the PKD1-like duplications made the likelihood of piecing together a full-length PKD1 transcript by merely screening cDNA libraries via hybridization very low. Such a

screening method would be as likely to identify transcripts originating from both the PKD1-like duplicated regions as from the authentic PKD1 locus. In fact, if each of the duplicated loci were as transcriptionally active as the auhentic PKD1 locus, the representation of authentic PKD1 cDNA clones among the total positive clones, would be very low.

Thus, a strategy was developed for obtaining the authentic PKD1 sequence which included, first, a plan for obtaining the highest quality of both genomic sequence spanning the duplicated region as well as obtaining duplicate coverage of cDNA sequence spanning the expected length of the PKD1 transcript; second, to compare the cDNA sequences to the genomic sequence spanning the duplicated region, thus identiying PKD1 exons; and, finally, to assemble the identified exons into a full-length PKD1 coding sequence. The isolation of both PKD1 genomic and cDNA sequence and, further, the aligning of such sequences, however, proved to be very difficult.

PKD1 genomic DNA (whch totals approximately 60 kb) proved to be particularly difficult to characterize for a number of reasons. First, portions of PKD1 genomic DNA (specifically, regions within cosmid cGGG10) tended to be preferentially subcloned. For example, screens for trinucleotide repeats in the cGGG10 cosmid identified one CCT-positive subclone in a Sau3A-generated library of cGGG10 sublcones. This region was, however, vastly underrepresented in both the Sau3A library (i.e., approximately 1 clone out of over 10,000) and subsequent sheared cosmid libraries (in which no such clones were isolated). A plasmid sublone containing the region, G13, proved difficult to grow and to sequence. Sequence analysis of the clone revealed a highly monotonous series of purines (A and G). Such sequences are thought to make the clone difficult to stably propagate in bacteria. Thus, in order to ascertain the level of representation of the cosmid, it was necessary to construct a detailed physical map of the cGGG10 cosmid.

Second, genomic sequence within the PKD1 region is very GC-rich (approximately 70%), and forms extensive, stable secondary structures. These PKD1 genomic DNA features made the task of obtaining accurate nucleotide sequence very difficult. Several alternative sequencing conditions, including different polymerases, melting conditions, polymerization conditons and combinations thereof had to be utilized before such sequence was obtained. However, even when reliable nucleotide sequence became available, the extensive amount of repeated sequences within the genomic made the aligning of sequence information very difficult. It became necesary for accurate aligning of sequences, therefore, to use the fine physical map which had been created earlier.

The sequencing of PKD1 cDNA also presented a number of PKD1-specific difficulties. First, the 14 kb size of the transcript made it impossible to isolate a single cDNA clone containg the entire PKD1 transcript. Overlapping partial cDNA clones, therefore, had to be obtained in order to piece together an entire sequence. Partial cDNA clones were obtained by sequencing the ends of one cDNA insert, synthesizing probes using this sequence, and obtaining overlapping cDNA clones by their hybridization to such probes. Second, the PKD1 gene was poorly represented in renal cDNA libraries, and, in fact, its expression appeared to be low in a number of tissues, making the isolation of PKD1 cDNA clones especially difficult.

5.1.3. THE PKD1 GENE

Described, herein is the complete nucleotide sequence of the extensive PKD1 gene coding region. Further, PKD1 alternative splicing features are discussed, below.

The coding region of the PKD1 gene is complex and extensive, containing a total of 46 exons and producing a transcript of approximately 14 kb in length. FIG. 5A depicts the structure of the PKD1 gene transcript. A total of 46 exons were identified within the PKD1 gene. Additionally,

sequence analysis from a number of cDNA clones reveals that the gene may have alternatively spliced forms. FIGS. 5B-5C show a table of exons, listing exon sizes and indicating which cDNA clones contain nucleotide sequences corresponding to sequences present within specific exons.

FIGS. 6A-6P depict the PKD1 nucleotide sequence. Specifically, the top line of FIGS. 6A-6P shows the nucleotide sequence of the entire PKD1 coding region (SEQ ID NO: 1). The term "PKD1 gene", as used herein, refers to (a) the nucleotide sequence depicted in FIGS. 6A-6P (SEQ ID NO: 1); (b) any DNA sequence that hybridizes to the complement of the nucleotide sequence depicted in FIGS. 6A-6P (SEQ ID NO: 1), under highly stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and which encodes a gene product functionally equivalent to the PKD1 gene product (SEQ ID NO: 2) depicted in FIGS. 6A-6P ; and/or (c) any DNA sequence that hybridizes to the complement of the nucleotide sequence depicted in FIGS. 6A-6P (SEQ ID NO: 1) under less stringent conditions, such as moderately stringent conditions, e.g., washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989, *supra*), yet which still encodes a gene product functionally equivalent to the PKD1 gene product depicted in FIGS. 6A-6P (SEQ ID NO: 2).

The term "functionally equivalent" as used herein can refer to: 1) a gene product or peptide having the biological function of the PKD1 gene product depicted in FIGS. 6A-6P and/or the biological function of a PKD1 peptide domain, as depicted in FIGS. 7A-7B and 8; 2) a gene product containing at least one PKD1 peptide domain as depicted in FIGS. 7A-7B and 8; or 3) a gene product having an 80% overall amino acid residue similarity to the PKD1 gene product depicted in FIGS. 6A-6P. The term "functionally equivalent gene" as used herein can further refer a nucleotide sequence which encodes

a gene product of 1, 2 or 3, as described earlier in this paragraph.

The invention also includes nucleic acid molecules, preferably DNA molecules, that hybridize to, and are therefore the complements of, the DNA sequences (a) through (c), in the preceding paragraph. Such hybridization conditions may be highly stringent or less highly stringent, as described above. In instances wherein the nucleic acid molecules are oligonucleotides ("oligos"), highly stringent conditions may refer, e.g., to washing in 6xSSC/0.05% sodium pyrophosphate at 37°C (for 14-base oligos), 48°C (for 17-base oligos), 55°C (for 20-base oligos), and 60°C (for 23-base oligos). These nucleic acid molecules may act as PKD1 antisense molecules, useful, for example, in PKD1 gene regulation and/or as antisense primers in amplification reactions of PKD1 nucleic acid sequences. Further, such sequences may be used as part of ribozyme and/or triple helix sequences, also useful for PKD gene regulation. Still further, such molecules may be used as components of diagnostic methods whereby the level of PKD1 transcript may be deduced and/or the presence of an ADPKD-causing allele may be detected. Further, such sequences can be used to screen for and identify PKD1 homologs from, for example, other species.

The invention also encompasses (a) DNA vectors that contain any of the foregoing coding sequences and/or their complements (i.e., antisense); (b) DNA expression vectors that contain any of the foregoing coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences; and (c) genetically engineered host cells that contain any of the foregoing coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell. As used herein, regulatory elements include but are not limited to inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression.

For example, such regulatory elements may include CMV immediate early gene regulatory sequences, SV40 early or late promoter sequences on adenovirus, lac system, trp system, tac system or the trc system sequences. The invention includes fragments of any of the DNA sequences disclosed herein.

In addition to the PKD1 gene sequences described above, homologs of the PKD1 gene of the invention, as may, for example be present in other, non-human species, may be identified and isolated by molecular biological techniques well known in the art and, for example, labelled probes of small as 12 bp. Further, mutant PKD1 alleles and additional normal alleles of the human PKD1 gene of the invention, may be identified using such techniques. Still further, there may exist genes at other genetic loci within the human genome that encode proteins which have extensive homology to one or more domains of the PKD1 gene product. Such genes may also be identified via such techniques.

For example, such a previously unknown PKD1-type gene sequence may be isolated by performing a polymerase chain reaction (PCR; the experimental embodiment set forth by Mullis, K.B., 1987, U.S. Patent No. 4,683,202) using two degenerate oligonucleotide primer pools designed on the basis of amino acid sequences within the PKD1 gene described herein (see, e.g. FIGS. 6A-6P, SEQ ID NO: 2). The template for the reaction may be cDNA obtained by reverse transcription of mRNA prepared from human or non-human cell lines or tissue known to express a PKD1 allele or PKD1 homologue. The PCR product may be subcloned and sequenced to insure that the amplified sequences represent the sequences of a PKD1 or a PKD-like nucleic acid sequence. The PCR fragment may then be used to isolate a full length PKD1 cDNA clone by radioactively labeling the amplified fragment and screening a bacteriophage cDNA library. Alternatively, the labeled fragment may be used to screen a genomic library. For a review of cloning strategies which may be used, see e.g., Maniatis, 1989, Molecular Cloning, A Laboratory Manual, Cold Springs Harbor Press, N.Y.; and Ausubel et al., 1989, Current

Protocols in Molecular Biology, (Green Publishing Associates and Wiley Interscience, N.Y.).

5.2. THE PKD1 GENE PRODUCT

The PKD1 gene products of the invention include the PKD1 gene product encoded by the PKD1 nucleotide sequence depicted in FIGS. 6A-6P (SEQ ID NO: 2). The PKD1 gene product shown in FIGS. 6A-6P is a protein of 4304 amino acid residues, with a predicted mass of approximately 467 kilodaltons. This PKD1 gene product contains as least five distinct peptide domains which are likely to be involved in protein-protein and/or protein-carbohydrate interactions. Further, this PKD1 gene product shares amino acid sequence similarity with a number of extracellular matrix proteins. (See FIGS. 7A-7B and 8, which list the PKD1 gene product domains.) The PKD1 gene product domains are more fully described below, in the Example presented in Section 10.

In addition, PKD1 gene products that represent functionally equivalent gene products are within the scope of the invention. "Functionally equivalent" as used herein is as defined in Section 5.1, above. Such an equivalent PKD1 gene product may contain deletions, additions or substitutions of amino acid residues within the PKD1 sequence encoded by the PKD1 gene sequences described, above, in Section 5.1.3, but which result in a silent change thus producing a functionally equivalent PKD1 protein. Such amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipatic nature of the residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups having similar hydrophilicity values include the following: leucine, isoleucine, valine, glycine, analine, asparagine, glutamine, serine, threonine, phenylalanine and tyrosine. As used herein, a functionally equivalent PKD1 refers to a protein that exhibits substantially the same

biological activity as the PKD1 gene product encoded by the PKD1 gene sequences described in Section 5.1.1, above.

PKD1 gene products and peptides substantially similar to the PKD1 gene product encoded by the PKD1 gene sequences described in Section 5.1, above, which cause ADPKD symptoms are also intended to fall within the scope of the invention. Such gene products and peptides may include dominant mutant PKD1 gene products, or PKD1 gene products functionally equivalent to such mutant PKD1 gene products. By "functionally equivalent mutant PKD1 gene product" it is meant PKD1-like proteins that exhibit a biological activity substantially similar to the activity demonstrated by dominant mutant PKD1 gene products.

The PKD1 wild type or mutant protein may be purified from natural sources, as discussed in Section 5.2.1, below, or may, alternatively, be chemically synthesized or recombinantly expressed, as discussed in Section 5.2.2, below.

5.2.1 PKD1 PROTEIN PURIFICATION METHODS

The PKD1 protein may be substantially purified from natural sources (e.g., purified from cells) using protein separation techniques well known in the art. "Substantially purified" signifies purified away from at least about 90% (on a weight basis), and from at least about 99% of other proteins, glycoproteins, and other macromolecules normally found in such natural sources.

Such purification techniques may include, but are not limited to ammonium sulfate precipitation, molecular sieve chromatography, and/or ion exchange chromatography. Alternatively, or additionally, the PKD1 gene product may be purified by immunoaffinity chromatography using an immunoabsorbent column to which an antibody is immobilized which is capable of binding the PKD1 gene product. Such an antibody may be monoclonal or polyclonal in origin. If the PKD1 gene product is specifically glycosylated, the glycosylation pattern may be utilized as part of a

purification scheme via, for example, lectin chromatography.

The cellular sources from which the PKD1 gene product may be purified may include, but are not limited to, those cells that are expected, by Northern and/or Western blot analysis, to express the PKD1 gene. Preferably, such cellular sources are renal tubular epithelial cells, bilary duct cells, skeletal muscle cells, whole brain cells, lung alveolar epithelial cell, and placental cell.

One or more forms of the PKD1 gene product may be secreted out of the cell, i.e., may be extracellular. Such extracellular forms of the PKD1 gene product may preferably be purified from whole tissue rather than cells, utilizing any of the techniques described above. Preferable tissue includes, but is not limited to those tissues than contain cell types such as those described above. Alternatively, PKD1 expressing cells such as those described above may be grown in cell culture, under conditions well known to those of skill in the art. The PKD1 gene product may then be purified from the cell media using any of the techniques discussed above.

5.2.2. PKD1 PROTEIN SYNTHESIS AND EXPRESSION METHODS

Methods for the chemical synthesis of polypeptides (e.g., gene products) or fragments thereof, are well-known to those of ordinary skill in the art, e.g., peptides can be synthesized by solid phase techniques, cleaved from the resin and purified by preparative high performance liquid chromatography (see, e.g., Creighton, 1983, Proteins: Structures and Molecular Principles, W.H. Freeman & Co., N.Y., pp. 50-60). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing; e.g., using the Edman degradation procedure (see e.g., Creighton, 1983, *supra* at pp. 34-49). Thus, the PKD1 protein may be chemically synthesized in whole or in part.

The PKD1 protein may additionally be produced by recombinant DNA technology using the PKD1 nucleotide sequences as described, above, in Section 5.1, coupled with

techniques well known in the art. Thus, methods for preparing the PKD1 polypeptides and peptides of the invention by expressing nucleic acid encoding PKD1 sequences are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing PKD1 protein coding sequences and appropriate transcriptional/translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques and <u>in vivo</u> recombination/genetic recombination. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y., both of which are incorporated by reference herein in their entirety. Alternatively, RNA capable of encoding PKD1 protein sequences may be chemically synthesized using, for example, automated or semi-automated synthesizers. See, for example, the techniques described in "Oligonucleotide Synthesis", 1984, Gait, M.J. ed., IRL Press, Oxford, which is incorporated by reference herein in its entirety.

A variety of host-expression vector systems may be utilized to express the PKD1 coding sequences of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, exhibit the PKD1 protein of the invention <u>in situ</u>. These include but are not limited to microorganisms such as bacteria (<u>e.g.</u>, <u>E. coli</u>, <u>B. subtilis</u>) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing PKD1 protein coding sequences; yeast (<u>e.g.</u>, <u>Saccharomyces</u>, <u>Pichia</u>) transformed with recombinant yeast expression vectors containing the PKD1 protein coding sequences; insect cell systems infected with recombinant virus expression vectors (<u>e.g.</u>, baculovirus) containing the PKD1 protein coding

sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the PKD1 protein coding sequences coding sequence; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the PKD1 protein being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of antibodies or to screen peptide libraries, for example, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited to, the E. coli expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the PKD1 protein coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned PKD1 protein can be released from the GST moiety.

In an insect system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda

cells. The PKD1 coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of PKD1 coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect Spodoptera frugiperda cells in which the inserted gene is expressed (e.g., see Smith et al., 1983, J. Viol. 46:584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the PKD1 coding sequence or interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing PKD1 protein in infected hosts (e.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted PKD1 coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire PKD1 gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the PKD1 coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both

natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cells lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, etc.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the PKD1 protein may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the PKD1 protein. Such

engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the PKD1 protein.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes.

Whether produced by molecular cloning methods or by chemical synthetic methods, the amino acid sequence of the PKD1 protein which may be used in the assays of the invention need not be identical to the amino acid sequence encoded by the PKD1 gene reported herein. The PKD1 proteins or peptides used may comprise altered sequences in which amino acid residues are deleted, added, or substituted, while still resulting in a gene product functionally equivalent to the PKD1 gene product. "Functionally equivalent", as utilized herein, is as defined, above, in Section 5.1, and is additionally defined to refer to peptides capable of interacting with other cellular or extracellular molecules in a manner substantially similar to the way in which the corresponding portion of the endogenous PKD1 gene product would.

For example, functionally equivalent amino acid residues may be substituted for residues within the sequence resulting

in a change of amino acid sequence. Such substitutes may be selected from other members of the class (i.e., non-polar, positively charged or negatively charged) to which the amino acid belongs; e.g., the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; the polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; the positively charged (basic) amino acids include arginine, lysine, and histidine; the negatively charged (acidic) amino acids include aspartic and glutamic acid.

When used as a component in the assay systems described herein, the PKD1 gene product or peptide (e.g., gene product fragment) may be labeled, either directly or indirectly, to facilitate detection of a complex formed between the PKD1 gene product and a test substance. Any of a variety of suitable labeling systems may be used including but not limited to radioisotopes such as ^{125}I; enzyme labelling systems that generate a detectable colorimetric signal or light when exposed to substrate; and fluorescent labels.

Where recombinant DNA technology is used to produce the PKD1 protein for the assay systems described herein, it may be advantageous to engineer fusion proteins that can facilitate labeling, immobilization and/or detection. For example, the coding sequence of the viral or host cell protein can be fused to that of a heterologous protein that has enzyme activity or serves as an enzyme substrate in order to facilitate labeling and detection. The fusion constructs should be designed so that the heterologous component of the fusion product does not interfere with binding of the host cell and viral protein.

Indirect labeling involves the use of a third protein, such as a labeled antibody, which specifically binds to one of the binding partners, i.e., either the PKD1 protein or its binding partner used in the assay. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric,

single chain, Fab fragments and fragments produced by an Fab expression library.

5.3. ANTIBODIES REACTIVE WITH PKD1 GENE PRODUCT

Described herein are methods for the production of antibodies capable of specifically recognizing one or more PKD1 gene product epitopes. Such antibodies may include, but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, $F(ab')_2$ fragments, fragments produced by a FAb expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Such antibodies may be used, for example, in the detection of PKD1 gene product in a biological sample, or, alternatively, as a method for the inhibition of abnormal PKD1 activity. Thus, such antibodies may be utilized as part of ADPKD treatment methods, and/or may be used as part of diagnostic techniques whereby patients may be tested for abnormal levels of PKD1 gene product, or for the presence of abnormal forms of the PKD1 protein.

For the production of antibodies to PKD1, various host animals may be immunized by injection with PKD1 protein, or a portion thereof. Such host animals may include but are not limited to, rabbits, mice, and rats.. Various adjuvants may be used to increase the immunological response, depending on the host species, including, but not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacteriumparvum.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, such as PKD1, or an antigenic functional derivative thereof. For the production of polyclonal antibodies, host animals such as those described above, may

be immunized by injection with PKD1 protein supplemented with adjuvants as also described above.

Monoclonal antibodies which are substantially homogeneous populations of antibodies to a particular antigen, may be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein (1975, Nature 256:495-497; and U.S. Patent No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72; Cole et al., 1983, Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class, including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454; U.S. Patent No. 4,816,567, which is incorporated by reference herein in its entirety) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a murine variable region and a human immunoglobulin constant region.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; Bird, 1988, Science 242:423-426; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 334:544-546) can be adapted to produce PKD1-single chain antibodies. Single chain antibodies are formed by linking

the heavy and light chain fragment of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Further, PKD1-humanized monoclonal antibodies may be produced using standard techniques (see, for example, U.S. Patent No. 5,225,539, which is incorporated herein by reference in its entirety).

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

5.4. SCREENING ASSAYS FOR COMPOUNDS THAT INTERACT WITH THE PKD1 GENE PRODUCT

The following assays are designed to identify compounds that bind to the PKD1 gene product; other cellular proteins that interact with the PKD1 gene product; and compounds that interfere with the interaction of the PKD1 product with other cellular proteins.

Compounds identified via assays such as those described herein may be useful, for example, in elaborating the biological function of the PKD1 gene product, and for ameliorating ADPKD symptoms caused by mutations within the PKD1 gene. In instances whereby a mutation with the PKD1 gene causes a lower level of expression, and therefore results in an overall lower level of PKD1 activity in a cell or tissue, compounds that interact with the PKD1 gene product may include ones which accentuate or amplify the activity of the bound PKD1 protein. Thus, such compounds would bring about an effective increase in the level of PKD1 activity, thus ameliorating ADPKD symptoms. In instances whereby mutations with the PKD1 gene cause aberrant PKD1 proteins to

be made which have a deleterious effect that leads to ADPKD, compounds that bind PKD1 protein may be identified that inhibit the activity of the bound PKD1 protein.

This decrease in the aberrant PKD1 activity can therefore, serve to ameliorate ADPKD symptoms. Assays for testing the effectiveness of compounds, identified by, for example, techniques such as those described in this Section are discussed, below, in Section 5.3.

5.5. IN VITRO SCREENING ASSAYS FOR COMPOUNDS THAT BIND TO THE PKD1 PROTEIN

In vitro systems may be designed to identify compounds capable of binding the PKD1 gene of the invention. Such compounds may include, but are not limited to, peptides made of D-and/or L-configuration amino acids (in, for example, the form of random peptide libraries; see Lam, K.S. *et al*., 1991, Nature 354:82-84), phosphopeptides (in, for example, the form of random or partially degenerate, directed phosphopeptide libraries; see, for example, Songyang, Z. *et al*., 1993, Cell 72:767-778), antibodies, and small or large organic or inorganic molecules. Compounds identified may be useful, for example, in modulating the activity of PKD1 proteins, preferably mutant PKD1 proteins, may be useful in elaborating the biological function of the PKD1 protein, may be utilized in screens for identifying compounds that disrupt normal PKD1 interactions, or may in themselves disrupt such interactions.

The principle of the assays used to identify compounds that bind to the PKD1 protein involves preparing a reaction mixture of the PKD1 protein and the test compound under conditions and for a time sufficient to allow the two components to interact and bind, thus forming a complex which can be removed and/or detected in the reaction mixture. These assays can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays involve anchoring PKD1 or the test substance onto a solid phase and detecting PKD1/test substance complexes anchored on the solid phase at the end of the reaction. In homogeneous assays, the entire

reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested.

In a heterogeneous assay system, the PKD1 protein may be anchored onto a solid surface, and the test substance, which is not anchored, is labeled, either directly or indirectly. In practice, microtiter plates are conveniently utilized. The anchored component may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished simply by coating the solid surface with a solution of the protein and drying. Alternatively, an immobilized antibody, preferably a monoclonal antibody, specific for the protein may be used to anchor the protein to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the labeled component is added to the coated surface containing the anchored component. After the reaction is complete, unreacted components are removed (e.g., by washing) under conditions such that any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the labeled compound is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the labeled component is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the binding partner (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody).

Alternatively, a heterogenous reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected; e.g., using an immobilized antibody specific for PKD1 or the test substance to anchor any complexes formed in solution, and a labeled

antibody specific for the other binding partner to detect anchored complexes.

In an alternate embodiment of the invention, a homogeneous assay can be used. In this approach, a preformed complex of the PKD1 protein and a known binding partner is prepared in which one of the components is labeled, but the signal generated by the label is quenched due to complex formation (see, e.g., U.S. Patent No. 4,109,496 by Rubenstein which utilizes this approach for immunoassays). The addition of a test substance that competes with and displaces one of the binding partners from the preformed complex will result in the generation of a signal above background.

5.6. ASSAYS FOR CELLULAR PROTEINS THAT INTERACT WITH PKD1 PROTEIN

Any method suitable for detecting protein-protein interactions may be employed for identifying novel PKD1-cellular or extracellular protein interactions. For example, some traditional methods which may be employed are co-immunoprecipitation, crosslinking and copurification through gradients or chromatographic columns. Additionally, methods which result in the simultaneous identification of the genes coding for the protein interacting with a target protein may be employed. These methods include, for example, probing expression libraries with labeled target protein, using this protein in a manner similar to antibody probing of λgt11 libraries.

One such method which detects protein interactions *in vivo*, the yeast two-hybrid system, is described in detail for illustration only and not by way of limitation. One version of this system has been described (Chien et al., 1991, Proc. Natl. Acad. Sci. USA, 88:9578-9582) and is commercially available from Clontech (Palo Alto, CA).

Briefly, utilizing such a system, plasmids are constructed that encode two hybrid proteins: one consists of the DNA-binding domain of a transcription activator protein fused to one test protein "X" and the other consists of the

activator protein's activation domain fused to another test protein "Y". Thus, either "X" or "Y" in this system may be wild type or mutant PKD1, while the other may be a test protein or peptide. The plasmids are transformed into a strain of the yeast <u>Saccharomyces cerevisiae</u> that contains a reporter gene (<u>e.g.</u>, <u>lacZ</u>) whose regulatory region contains the activator's binding sites. Either hybrid protein alone cannot activate transcription of the reporter gene, the DNA-binding domain hybrid because it does not provide activation function and the activation domain hybrid because it cannot localize to the activator's binding sites. Interaction of the two proteins reconstitutes the functional activator protein and results in expression of the reporter gene, which is detected by an assay for the reporter gene product.

The two-hybrid system or related methodology can be used to screen activation domain libraries for proteins that interact with a PKD1 protein. Total genomic or cDNA sequences are fused to the DNA encoding an activation domain. This library and a plasmid encoding a hybrid of the PKD1 protein fused to the DNA-binding domain are cotransformed into a yeast reporter strain, and the resulting transformants are screened for those that express the reporter gene. These colonies are purified and the plasmids responsible for reporter gene expression are isolated. DNA sequencing is then used to identify the proteins encoded by the library plasmids.

For example, and not by way of limitation, the PKD1 gene can be cloned into a vector such that it is translationally fused to the DNA encoding the DNA-binding domain of the GAL4 protein. A cDNA library of the cell line from which proteins that interact with PKD1 are to be detected can be made using methods routinely practiced in the art. According to this particular system, for example, the cDNA fragments can be inserted into a vector such that they are translationally fused to the activation domain of GAL4. This library can be co-transformed along with the PKD1-GAL4 DNA binding domain fusion plasmid into a yeast strain which contains a <u>lacZ</u> gene

driven by a promoter which contains GAL4 activation sequences. A cDNA encoded protein, fused to GAL4 activation domain, that interacts with PKD1 will reconstitute an active GAL4 protein and thereby drive expression of the lacZ gene. Colonies which express lacZ can be detected by their blue color in the presence of X-gal. The cDNA can then be extracted from strains derived from these and used to produce and isolate the PKD1-interacting protein using techniques routinely practiced in the art.

5.7. ASSAYS FOR COMPOUNDS THAT INTERFERE WITH PKD1/CELLULAR PROTEIN INTERACTION

The PKD1 protein of the invention may, *in vivo*, interact with one or more cellular or extracellular proteins. Such cellular proteins are referred to herein as "binding partners". Compounds that disrupt such interactions may be useful in regulating the activity of the PKD1 protein, especially mutant PKD1 proteins. Such compounds may include, but are not limited to molecules such as antibodies, peptides, and the like described in Section 5.2.1. above.

In instances whereby ADPKD symptoms are caused by a mutation within the PKD1 gene which produces PKD1 gene products having aberrant, gain-of-function activity, compounds identified that disrupt such interactions may, therefore inhibit the aberrant PKD1 activity. Preferably, compounds may be identified which disrupt the interaction of mutant PKD1 gene products with cellular or extracellular proteins, but do not substantially effect the interactions of the normal PKD1 protein. Such compounds may be identified by comparing the effectiveness of a compound to disrupt interactions in an assay containing normal PKD1 protein to that of an assay containing mutant PKD1 protein.

The basic principle of the assay systems used to identify compounds that interfere with the interaction between the PKD1 protein, preferably mutant PKD1 protein, and its cellular or extracellular protein binding partner or partners involves preparing a reaction mixture containing the

PKD1 protein and the binding partner under conditions and for a time sufficient to allow the two proteins to interact and bind, thus forming a complex. In order to test a compound for inhibitory activity, the reaction is conducted in the presence and absence of the test compound, i.e., the test compound may be initially included in the reaction mixture, or added at a time subsequent to the addition of PKD1 and its cellular or extracellular binding partner; controls are incubated without the test compound or with a placebo. The formation of any complexes between the PKD1 protein and the cellular or extracellular binding partner is then detected. The formation of a complex in the control reaction, but not in the reaction mixture containing the test compound indicates that the compound interferes with the interaction of the PKD1 protein and the interactive protein. As noted above, complex formation within reaction mixtures containing the test compound and normal PKD1 protein may also be compared to complex formation within reaction mixtures containing the test compound and mutant PKD1 protein. This comparison may be important in those cases wherein it is desirable to identify compounds that disrupt interactions of mutant but not normal PKD1 proteins.

The assay for compounds that interfere with the interaction of the binding partners can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays involve anchoring one of the binding partners onto a solid phase and detecting complexes anchored on the solid phase at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds that interfere with the interaction between the binding partners, e.g., by competition, can be identified by conducting the reaction in the presence of the test substance; i.e., by adding the test substance to the reaction mixture prior to or simultaneously with the PKD1 protein and interactive cellular or

extracellular protein. On the other hand, test compounds that disrupt preformed complexes, e.g. compounds with higher binding constants that displace one of the binding partners from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. The various formats are described briefly below.

In a heterogeneous assay system, one binding partner, e.g., either the PKD1 protein or the interactive cellular or extracellular protein, is anchored onto a solid surface, and its binding partner, which is not anchored, is labeled, either directly or indirectly. In practice, microtiter plates are conveniently utilized. The anchored species may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished simply by coating the solid surface with a solution of the protein and drying. Alternatively, an immobilized antibody specific for the protein may be used to anchor the protein to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the binding partner of the immobilized species is added to the coated surface with or without the test compound. After the reaction is complete, unreacted components are removed (e.g., by washing) and any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the binding partner was pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the binding partner is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the binding partner (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody). Depending upon the order of addition of reaction components, test compounds which inhibit complex formation or which disrupt preformed complexes can be detected.

Alternatively, the reaction can be conducted in a liquid phase in the presence or absence of the test compound, the

reaction products separated from unreacted components, and complexes detected; e.g., using an immobilized antibody specific for one binding partner to anchor any complexes formed in solution, and a labeled antibody specific for the other binding partner to detect anchored complexes. Again, depending upon the order of addition of reactants to the liquid phase, test compounds which inhibit complex or which disrupt preformed complexes can be identified.

In an alternate embodiment of the invention, a homogeneous assay can be used. In this approach, a preformed complex of the PKD1 protein and the interactive cellular or extracellular protein is prepared in which one of the binding partners is labeled, but the signal generated by the label is quenched due to complex formation (see, e.g., U.S. Patent No. 4,109,496 by Rubenstein which utilizes this approach for immunoassays). The addition of a test substance that competes with and displaces one of the binding partners from the preformed complex will result in the generation of a signal above background. In this way, test substances which disrupt PKD1 protein-cellular or extracellular protein interaction can be identified.

In a particular embodiment, the PKD1 protein can be prepared for immobilization using recombinant DNA techniques described in Section 5.1.2.2, *supra*. For example, the PKD1 coding region can be fused to the glutathione-S-transferase (GST) gene using the fusion vector pGEX-5X-1, in such a manner that its binding activity is maintained in the resulting fusion protein. The interactive cellular or extracellular protein can be purified and used to raise a monoclonal antibody, using methods routinely practiced in the art and described above. This antibody can be labeled with the radioactive isotope ^{125}I, for example, by methods routinely practiced in the art. In a heterogeneous assay, e.g., the GST-PKD1 fusion protein can be anchored to glutathione-agarose beads. The interactive cellular or extracellular protein can then be added in the presence or absence of the test compound in a manner that allows

interaction and binding to occur. At the end of the reaction period, unbound material can be washed away, and the labeled monoclonal antibody can be added to the system and allowed to bind to the complexed binding partners. The interaction between the PKD1 protein and the interactive cellular or extracellular protein can be detected by measuring the amount of radioactivity that remains associated with the glutathione-agarose beads. A successful inhibition of the interaction by the test compound will result in a decrease in measured radioactivity.

Alternatively, the GST-PKD1 fusion protein and the interactive cellular or extracellular protein can be mixed together in liquid in the absence of the solid glutathione-agarose beads. The test compound can be added either during or after the binding partners are allowed to interact. This mixture can then be added to the glutathione-agarose beads and unbound material is washed away. Again the extent of inhibition of the binding partner interaction can be detected by adding the labeled antibody and measuring the radioactivity associated with the beads.

In another embodiment of the invention, these same techniques can be employed using peptide fragments that correspond to the binding domains of the PKD1 protein and the interactive cellular or extracellular protein, respectively, in place of one or both of the full length proteins. Any number of methods routinely practiced in the art can be used to identify and isolate the protein's binding site. These methods include, but are not limited to, mutagenesis of one of the genes encoding the proteins and screening for disruption of binding in a co-immunoprecipitation assay. Compensating mutations in the PKD1 gene can be selected. Sequence analysis of the genes encoding the respective proteins will reveal the mutations that correspond to the region of the protein involved in interactive binding. Alternatively, one protein can be anchored to a solid surface using methods described in this Section above, and allowed to interact with and bind to its labeled binding partner, which

has been treated with a proteolytic enzyme, such as trypsin. After washing, a short, labeled peptide comprising the binding domain may remain associated with the solid material, which can be isolated and identified by amino acid sequencing. Also, once the gene coding for the for the cellular or extracellular protein is obtained, short gene segments can be engineered to express peptide fragments of the protein, which can then be tested for binding activity and purified or synthesized.

For example, and not by way of limitation, PKD1 can be anchored to a solid material as described above in this section by making a GST-PKD1 fusion protein and allowing it to bind to glutathione agarose beads. The interactive cellular protein can be labeled with a radioactive isotope, such as ^{35}S, and cleaved with a proteolytic enzyme such as trypsin. Cleavage products can then be added to the anchored GST-PKD1 fusion protein and allowed to bind. After washing away unbound peptides, labeled bound material, representing the cellular or extracellular protein binding domain, can be eluted, purified, and analyzed for amino acid sequence by methods described in Section 5.1.2.2, *supra*. Peptides so identified can be produced synthetically or fused to appropriate facilitative proteins using recombinant DNA technology, as described in Section 5.1.2.2, *supra*.

5.8. ASSAYS FOR ADKPD-INHIBITORY ACTIVITY

Any of the binding compounds, including but not limited to, compounds such as those identified in the foregoing assay systems may be tested for anti-ADPKD activity. ADPKD, an autosomal dominant disorder, may involve underexpression of a wild-type PKD1 allele, or expression of a PKD1 gene product that exhibits little or no PKD1 activity. In such an instance, even though the PKD1 gene product is present, the overall level of normal PKD1 gene product present is insufficient and leads to ADPKD symptoms. As such, "anti-ADPKD activity", as used herein, may refer to a increase in the level of expression of the normal PKD1 gene product, to

levels wherein ADPKD symptoms are ameliorated. Additionally, the term may refer to an increase in the level of normal PKD1 activity in the cell, to levels wherein ADPKD symptoms are ameliorated.

Alternatively, ADPKD may be caused by the production of an aberrant mutant form of the PKD1 protein, which either interferes with the normal allele product or introduces a novel function into the cell, which then leads to the mutant phenotype. For example, a mutant PKD1 protein may compete with the wild type protein for the binding of a substance required to relay a signal inside or outside of a cell. Circumstances such as these are referred to as "gain of function" mutations. It is possible that different mechanisms could be occurring in different patients which can lead to mutant phenotypic variations.

"Anti-ADPKD activity", as used herein, may refer to a decrease in the level and/or activity of such a mutant PKD1 protein so that symptoms of PKD1 are ameliorated.

Cell-based and animal model-based assays for the identification of compounds exhibiting anti-ADPKD activity are described below.

5.8.1. CELL BASED ASSAYS

Cells that contain and express mutant PKD1 gene sequences which encode mutant PKD1 protein, and thus exhibit cellular phenotypes associated with ADPKD, may be utilized to identify compounds that possess anti-ADPKD activity. Such cells may include cell lines consisting of naturally occurring or engineered cells which express mutant or express both normal and mutant PKD1 gene products. Such cells include, but are not limited to renal epithelial cells, including primary and immortalized human renal tubular cells, MDCK cells, LLPCK1 cells, and human renal carcinoma cells.

Cells, such as those described above, which exhibit ADPKD-like cellular phenotypes, may be exposed to a compound suspected of exhibiting anti-ADPKD activity at a sufficient concentration and for a time sufficient to elicit such anti-

ADPKD1 activity in the exposed cells. After exposure, the cells are examined to determine whether one or more of the ADPKD-like cellular phenotypes has been altered to resemble a more wild type, non-ADPKD phenotype.

Among the cellular phenotypes which may be followed in the above assays are differences in the apical/basolateral distribution of membrane proteins. For example, normal (i.e., non-ADPKD) renal tubular cells in situ and in culture under defined conditions have a characteristic pattern of apical/basolateral distribution of cell surface markers. ADPKD renal cells, by contrast, exhibit a distribution pattern that reflects a partially reversed apical/basolateral polarity relative to the normal distribution. For example, sodium-potassium ATPase is found on the basolateral membranes of renal epithelial cells but is found on the apical surface of ADPKD epithelial cells, both in cystic epithelia in vivo and in ADPKD cells in culture (Wilson, et al., 1991, Am. J. Physiol. 260:F420-F430). Among the other markers which exhibit an alteration in polarity in normal versus ADPKD affected cells are the EGF receptor, which is normally located basolaterally, but in ADPKD cells is mislocated to the apical surface. Such a apical/basolateral marker distribution phenotype may be followed, for example, by standard immunohistology techniques using antibodies specific to the marker(s) of interest in conjunction with procedures that are well known to those of skill in the art.

Additionally, assays for the function of the PKD1 gene product can, for example, include a measure of extracellular matrix (ECM) components, such as proteoglycans, laminin, fibronectin and the like, in that studies in both ADPKD and in rat models of acquired cystic disease (Carone, F.A. et al., 1989, Kidney International 35:1034-1040) have shown alterations in such components. Thus, any compound which serves to create an extracellular matrix environment which more fully mimics the normal ECM should be considered as a candidate for testing for an ability to ameliorate ADPKD symptoms.

5.8.2 ANIMAL MODEL ASSAYS

The ability of a compound, such as those identified in the foregoing binding assays, to prevent or inhibit disease may be assessed in animal models for ADPKD. Several naturally-occurring mutations for renal cystic disease have been found in animals. While these are not perfect models of ADPKD, they provide test systems for assaying the effects of compounds that interact with PKD1 proteins. Of these models, the Han:SPRD rat model is the only autosomal dominant example. Such a model is well known to those of skill in the art. See, for example, Kaspareit-Rittinghausen et al., 1989, Vet. Path. 26:195. In addition, several recessive models exist (Reeders, S., 1992, Nature Genetics 1:235).

Additionally, animal models exhibiting ADPKD-like symptoms may be engineered by utilizing PKD1 sequences such as those described, above, in Section 5.1, in conjunction with techniques for producing transgenic animals that are well known to those of skill in the art.

Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, and non-human primates, e.g., baboons, squirrels, monkeys, and chimpanzees may be used to generate such ADPKD animal models.

In instances wherein the PKD1 mutation leading to ADPKD symptoms causes a drop in the level of PKD1 protein or causes an ineffective PKD1 protein to be made (i.e., the PKD1 mutation is a dominant loss-of-function mutation) various strategies may be utilized to generate animal models exhibiting ADPKD-like symptoms. For example, PKD1 knockout animals, such as mice, may be generated and used to screen for compounds which exhibit an ability to ameliorate ADPKD systems. Animals may be generated whose cells contain one inactivated copy of a PKD1-homologue. In such a strategy, human PKD1 gene sequences may be used to identify a PKD1 homologue within the animal of interest, utilizing techniques described, above, in Section 5.1. Once such a PKD1 homologue has been identified, well-known techniques such as those

described, below, in Section 5.8.2.1. may be utilized to disrupt and inactivate the endogenous PKD1 homolog, and further, to produce animals which are heterozygous for such an inactivated PKD1 homolog. Such animals may then be observed for the development of ADPKD-like symptoms.

In instances wherein a PKD1 mutation causes a PKD1 protein having an aberrant PKD1 activity which leads to ADPKD symptoms (i.e., the PKD1 mutation is a dominant gain-of-function mutation) strategies such as those now described may be utilized to generate ADPKD animal models. First, for example, a human PKD1 gene sequence containing such a gain-of-function PKD1 mutation, and encoding such an aberrant PKD1 protein, may be introduced into the genome of the animal of interest by utilizing well known techniques such as those described, below, in Section 5.8.2.1. Such a PKD1 nucleic acid sequence must be controlled by a regulatory nucleic acid sequence which allows the mutant human PKD1 sequence to be expressed in the cells, preferably kidney cells, of the animal of interest. The human PKD1 regulatory promoter/enhancer sequences may be sufficient for such expression. Alternatively, the mutant PKD1 gene sequences may be controlled by regulatory sequences endogenous to the animal of interest, or by any other regulatory sequences which are effective in bringing about the expression of the mutant human PKD1 sequences in the animal cells of interest.

Expression of the mutant human PKD1 gene may be assayed, for example, by standard Northern analysis, and the production of the mutant human PKD1 gene product may be assayed by, for example, detecting its presence by utilizing techniques whereby binding of an antibody directed against the mutant human PKD1 gene product is detected. Those animals found to express the mutant human PKD1 gene product may then be observed for the development of ADPKD-like symptoms.

Alternatively, animal models of ADPKD may be produced by engineering animals containing mutations within one copy of their endogenous PKD1-homologue which correspond to gain-of-

function mutations within the human PKD1 gene. Utilizing such a strategy, a PKD1 homologue may be identified and cloned from the animal of interest, using techniques such as those described, above, in Section 5.1. One or more gain-of-function mutations may be engineered into such a PKD1 homolog which correspond to gain-of-function mutations within the human PKD1 gene. By "corresponding", it is meant that the mutant gene product produced by such an engineered PKD1 homologue will exhibit an aberrant PKD1 activity which is substantially similar to that exhibited by the mutant human PKD1 protein.

The engineered PKD1 homologue may then be introduced into the genome of the animal of interest, using techniques such as those described, below, in Section 5.8.2.1. Because the mutation introduced into the engineered PKD1 homologue is expected to be a dominant gain-of-function mutation, integration into the genome need not be via homologous recombination, although such a route is preferred.

Once transgenic animals have been generated, the expression of the mutant PKD1 homolog gene and protein may be assayed utilizing standard techniques, such as Northern and/or Western analyses. Animals expressing mutant PKD1 homolog proteins within the animals of interest, in cells or tissues, preferably kidney, of interest, the transgenic animals may be observed for the development of ADPKD-like symptoms.

Any of the ADPKD animal models described herein may be used to test compounds for an ability to ameliorate ADPKD symptoms.

In addition, as described in detail in Section 5.11 *infra*, such animal models can be used to determine the LD_{50} and the ED_{50} in animal subjects, and such data can be used to determine the in vivo efficacy of potential ADPKD treatments.

5.8.2.1 PRODUCTION OF PKD1 TRANSGENIC ANIMALS

Any technique known in the art may be used to introduce a PKD1 gene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to pronuclear microinjection (Hoppe, P.C. and Wagner, T.E., 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., 1985, Proc. Natl. Acad. Sci., USA 82:6148-6152); gene targeting in embryonic stem cells (Thompson et al., 1989, Cell 56:313-321); electroporation of embryos (Lo, 1983, Mol Cell. Biol. 3:1803-1814); and sperm-mediated gene transfer (Lavitrano et al., 1989, Cell 57:717-723); etc. For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115:171-229, which is incorporated by reference herein in its entirety).

When it is desired that the PKD1 transgene be integrated into the chromosomal site of the endogenous PKD1, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous PKD1 gene of interest are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of, the nucleotide sequence of the endogenous PKD1 gene.

Once the PKD1 founder animals are produced, they may be bred, inbred, outbred, or crossbred to produce colonies of the particular animal. Examples of such breeding strategies include but are not limited to: outbreeding of founder animals with more than one integration site in order to establish separate lines; inbreeding of separate lines in order to produce compound PKD1 transgenics that express the PKD1 transgene at higher levels because of the effects of additive expression of each PKD1 transgene; crossing of heterozygous transgenic animals to produce animals homozygous for a given integration site in order to both augment expression and eliminate the possible need for screening of animals by DNA analysis; crossing of separate homozygous

lines to produce compound heterozygous or homozygous lines; breeding animals to different inbred genetic backgrounds so as to examine effects of modifying alleles on expression of the PKD1 transgene and the development of ADPKD-like symptoms. One such approach is to cross the PKD1 founder animals with a wild type strain to produce an F1 generation that exhibits ADPKD symptoms, such as the development of polycystic kidneys. The F1 generation may then be inbred in order to develop a homozygous line, if it is found that homozygous PKD1 transgenic animals are viable.

The present invention provides for transgenic animals that carry the transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, i.e., mosaic animals. The transgene may be integrated as a single transgene or in concatamers, e.g., head-to-head tandems or head-to-tail tandems.

5.8.2.2. SELECTION AND CHARACTERIZATION OF THE PKD1 TRANSGENIC ANIMALS

The PKD1 transgenic animals that are produced in accordance with the procedures detailed, above, in Section 5.8.2.1., should be screened and evaluated to select those animals which may be used as suitable animal models for ADPKD.

Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to verify that integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, in situ hybridization analysis, and reverse transcriptase-PCR (rt-PCR). Samples of PKD1-expressing tissue, kidney tissue, for example, may be evaluated immunocytochemically using antibodies specific for the PKD1 transgene gene product.

The PKD1 transgenic animals that express PKD1 mRNA or gene product (detected immunocytochemically, using antibodies

directed against PKD1 tag epitopes) at easily detectable levels should then be further evaluated histopathologically to identify those animals which display characteristic ADPKD-like symptoms. Such transgenic animals serve as suitable model systems for ADPKD.

5.8.2.3. USES OF THE PKD1 ANIMAL MODELS

The PKD1 animal models of the invention may be used as model systems for ADPKD disorder and/or to generate cell lines that can be used as cell culture models for this disorder.

The PKD1 transgenic animal model systems for ADPKD may be used as a test substrate to identify drugs, pharmaceuticals, therapies and interventions which may be effective in treating such a disorder. Potential therapeutic agents may be tested by systemic or local administration. Suitable routes may include oral, rectal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, to name a few. The response of the animals to the treatment may be monitored by assessing the reversal of disorders associated with ADPKD. With regard to intervention, any treatments which reverse any aspect of ADPKD-like symptoms should be considered as candidates for human ADPKD therapeutic intervention. However, treatments or regimens which reverse the constellation of pathologies associated with any of these disorders may be preferred. Dosages of test agents may be determined by deriving dose-response curves, as discussed in Section 5.11, below.

In an alternate embodiment, the PKD1 transgenic animals of the invention may be used to derive a cell line which may be used as a test substrate in culture, to identify agents that ameliorate ADPKD-like symptoms. While primary cultures derived from the PKD1 transgenic animals of the invention may be utilized, the generation of continuous cell lines is

preferred. For examples of techniques which may be used to derive a continuous cell line from the transgenic animals, see Small *et al.*, 1985, Mol. Cell Biol. 5:642-648.

5.9. COMPOUNDS THAT INHIBIT EXPRESSION, SYNTHESIS OR ACTIVITY OF MUTANT PKD1 ACTIVITY

As discussed above, dominant mutations in the PKD1 gene that cause ADPKD may act as gain-of-function mutations which produce a form of the PKD1 protein which exhibits an aberrant activity that leads to the formation of ADPKD symptoms. A variety of techniques may be utilized to inhibit the expression, synthesis, or activity of such mutant PKD1 genes and gene products (*i.e.*, proteins).

For example, compounds such as those identified through assays described, above, in Section 5.4, which exhibit inhibitory activity, may be used in accordance with the invention to ameliorate ADPKD symptoms. Such molecules may include, but are not limited, to small and large organic molecules, peptides, and antibodies. Inhibitory antibody techniques are described, below, in Section 5.9.2.

Further, antisense and ribozyme molecules which inhibit expression of the PKD1 gene, preferably the mutant PKD1 gene, may also be used to inhibit the aberrant PKD1 activity. Such techniques are described, below, in Section 5.9.1. Still further, as described, below, in Section 5.9.1, triple helix molecules may be utilized in inhibiting the aberrant PKD1 activity.

5.9.1. INHIBITORY ANTISENSE, RIBOZYME AND TRIPLE HELIX APPROACHES

Among the compounds which may exhibit anti-ADPKD activity are antisense, ribozyme, and triple helix molecules. Such molecules may be designed to reduce or inhibit mutant PKD1 activity. Techniques for the production and use of such molecules are well known to those of skill in the art.

Antisense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. With respect to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between the -10 and +10 regions of the PKD1 nucleotide sequence of interest, are preferred.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by a endonucleolytic cleavage. The composition of ribozyme molecules must include one or more sequences complementary to the target PKD1 mRNA, preferably the mutant PKD1 mRNA, and must include the well known catalytic sequence responsible for mRNA cleavage. For this sequence, see U.S. Pat. No. 5,093,246, which is incorporated by reference herein in its entirety. As such, within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of RNA sequences encoding PKD1, preferably mutant PKD1 proteins.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequence: GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features, such as secondary structure, that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays.

Nucleic acid molecules to be used in triplex helix formation should be single stranded and composed of deoxynucleotides. The base composition of these oligonucleotides must be designed to promote triple helix

formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC' triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, contain a stretch of guanidine residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with one strand of a duplex first and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

It is possible that the antisense, ribozyme, and/or triple helix molecules described herein may reduce or inhibit the translation of mRNA produced by both normal and mutant PKD1 alleles. In order to ensure that substantial normal levels of PKD1 activity are maintained in the cell, nucleic acid molecules that encode and express PKD1 polypeptides exhibiting normal PKD1 activity may be introduced into cells which do not contain sequences susceptible to whatever antisense, ribozyme, or triple helix treatments. Such sequences may be introduced via gene therapy methods such as those described, below, in Section 5.5. Alternatively, it may be preferable to coadminister normal PKD1 protein into the cell or tissue in order to maintain the requisite level of cellular or tissue PKD1 activity.

below in Section 5.11 which are appropriate for peptide administration may be utilized to effectively administer inhibitory PKD1 antibodies to their site of action.

5.10 METHODS FOR RESTORING PKD1 ACTIVITY

As discussed above, dominant mutations in the PKD1 gene that cause ADPKD may lower the level of expression of the PKD1 gene or, alternatively, may cause inactive or substantially inactive PKD1 proteins to be formed. In either instance, the result is an overall lower level of normal PKD1 activity in the tissues or cells in which PKD1 is normally expressed. This lower level of PKD1 activity, then, leads to ADPKD symptoms. Thus, such PKD1 mutations represent dominant loss-of-function mutations. Described in this Section are methods whereby the level of normal PKD1 activity may be increased to levels wherein ADPKD symptoms are ameliorated.

For example, normal PKD1 protein, at a level sufficient to ameliorate ADPKD symptoms may be administered to a patient exhibiting such symptoms. Any of the techniques discussed, below, in Section 5.11, may be utilized for such administration. One of skill in the art will readily know how to determine the concentration of effective, non-toxic doses of the normal PKD1 protein, utilizing techniques such as those described, below, in Section 5.11.

Additionally, DNA sequences encoding normal PKD1 protein may be directly administered to a patient exhibiting ADPKD symptoms, at a concentration sufficient to produce a level of PKD1 protein such that ADPKD symptoms are ameliorated. Any of the techniques discussed, below, in Section 5.11, which achieve intracellular administration of compounds, such as, for example, liposome administration, may be utilized for the administration of such DNA molecules. The DNA molecules may be produced, for example, by recombinant techniques such as those described, above, in Section 5.1, and its subsections.

Further, patients with these types of mutations may be treated by gene replacement therapy. A copy of the normal PKD1 gene or a part of the gene that directs the production

of a normal PKD1 protein with the function of the PKD1 protein may be inserted into cells, renal cells, for example, using viral or non-viral vectors which include, but are not limited to vectors derived from, for example, retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, bovine papilloma virus or additional, non-viral vectors, such as plasmids. In addition, techniques frequently employed by those skilled in the art for introducing DNA into mammalian cells may be utilized. For example, methods including but not limited to electroporation, DEAE-dextran mediated DNA transfer, DNA guns, liposomes, direct injection, and the like may be utilized to transfer recombinant vectors into host cells. Alternatively, the DNA may be transferred into cells through conjugation to proteins that are normally targeted to the inside of a cell. For example, the DNA may be conjugated to viral proteins that normally target viral particles into the targeted host cell. Additionally, techniques such as those described in Sections 5.1 and 5.2 and their subsections, above, may be utilized for the introduction of normal PKD1 gene sequences into human cells.

The PKD1 gene is very large and, further, encodes a very large, approximately 14 kb, transcript. Additionally, the PKD1 gene product is large, having 4304 amino acids, with a molecular weight of about 467 kD. It is possible, therefore, that the introduction of the entire PKD1 coding region may be cumbersome and potentially inefficient as a gene therapy approach. However, because the entire PKD1 gene product may not be necessary to avoid the appearance of ADPKD symptoms, the use of a "minigene" therapy approach (see, e.g., Ragot, T. et al., 1993, Nature 361:647; Dunckley, M.G. et al., 1993, Hum. Mol. Genet. 2:717-723) can serve to ameliorate such ADPKD symptoms.

Such a minigene system comprises the use of a portion of the PKD1 coding region which encodes a partial, yet active or substantially active PKD1 gene product. As used herein, "substantially active" signifies that the gene product serves to ameliorate ADPKD symptoms. Thus, the minigene system

utilizes only that portion of the normal PKD1 gene which encodes a portion of the PKD1 gene product capable of ameliorating ADPKD symptoms, and may, therefore represent an effective and even more efficient ADPKD gene therapy than full-length gene therapy approaches. Such a minigene can be inserted into cells and utilized via the procedures described, above, for full-length gene replacement. The cells into which the PKD1 minigene are to be introduced are, preferably, those cells, such as renal cells, which are affected by ADPKD. Alternatively, any suitable cell can be transfected with a PKD1 minigene as long as the minigene is expressed in a sustained, stable fashion and produces a gene product that ameliorates ADPKD symptoms. Regulatory sequences by which such a PKD1 minigene can be successfully expressed will vary depending upon the cell into which the minigene is introduced. The skilled artisan will be aware of appropriate regulatory sequences for the given cell to be used. Techniques for such introduction and sustained expression are routine and are well known to those of skill in the art.

A therapeutic minigene for the amelioration of ADPKD symptoms can comprise a nucleotide sequence which encodes at least one PKD1 gene product peptide domain, as shown in FIGS. 7A-7B and 8. For example, such PKD1 peptide domains (the approximate amino acid residue positions of which are listed in parentheses after each domain name) can include a leucine-rich repeat domain (72 to 94, or 97 to 119) and/or a cysteine-rich repeat domain (32 to 65), a C-type (calcium dependent) lectin protein domain (405 to 534), an LDL-A module (641 to 671), one or more PKD domains (282 to 353; 1032 to 1124; 1138 to 1209; 1221 to 1292; 1305 to 1377; 1390 to 1463; 1477 to 1545; 1559 to 1629; 1643 to 1715; 1729 to 1799; 1815 to 1884; 1898 to 1968; 1983 to 2058; 2071 to 2142), or at least one C-terminal domain (2160 to 4304) (i.e., a peptide domain found in the C-terminal half of the PKD1 gene product). Minigenes which encode such PKD1 gene products can be synthesized and/or engineered using the PKD1

gene sequence (SEQ ID NO:1) disclosed herein, and by utilizing the amino acid residue domain designations found in FIGS. 7A-7B and 8.

Among the ways whereby the PKD1 minigene product activity can be assayed involves the use of PKD1 knockout animal models. Such animal models express an insufficient level of the PKD1 gene product. The production of such animal models may be as described above, in Section 5.8.2, and involves methods well known to those of skill in the art. PKD1 minigenes can be introduced into the PKD1 knockout animal models as, for example, described above, in this Section. The activity of the minigene can then be assessed by assaying for the amelioration of ADKPD-like symptoms. Thus, the relative importance of each of the PKD peptide domains, individually and/or in combination, with respect to PKD1 gene activity can be determined.

Cells, preferably, autologous cells, containing normal PKD1 expressing gene sequences may then be introduced or reintroduced into the patient at positions which allow for the amelioration of ADPKD symptoms. Such cell replacement techniques may be preferred, for example, when the PKD1 gene product is a secreted, extracellular gene product.

5.11. PHARMACEUTICAL PREPARATIONS AND METHODS OF ADMINISTRATION

The identified compounds that inhibit PKD1 expression, synthesis and/or activity can be administered to a patient at therapeutically effective doses to treat polycystic kidney disease. A therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms of polycystic kidney disease.

5.11.1. EFFECTIVE DOSE

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50}

(the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography. Additional factors which may be utilized to optimize dosage can include, for example, such factors as the severity of the ADPKD symptoms as well as the age, weight and possible additional disorders which the patient may also exhibit. Those skilled in the art will be able to determine the appropriate dose based on the above factors.

5.11.2. FORMULATIONS AND USE

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional

manner using one or more physiologically acceptable carriers or excipients.

Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycollate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g., gelatin, for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

5.12. DIAGNOSIS OF PKD1 ABNORMALITIES

A variety of methods may be employed, utilizing reagents such as PKD1 nucleotide sequences described in Sections 5.1, and antibodies directed against PKD1 gene product or peptides, as described, above, in Section 5.1.3. Specifically, such reagents may be used for the detection of the presence of PKD1 mutations, i.e., molecules present in diseased tissue but absent from, or present in greatly reduced levels relative to, the corresponding non-diseased tissue.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one specific PKD1 nucleic acid or anti-PKD1 antibody reagent described herein, which may be conveniently used, e.g., in clinical settings, to diagnose patients exhibiting PKD1 abnormalities.

Any tissue in which the PKD1 gene is expressed may be utilized in the diagnostics described below.

5.12.1 DETECTION OF PKD-1 NUCLEIC ACIDS

RNA from the tissue to be analyzed may be isolated using procedures which are well known to those in the art. Diagnostic procedures may also be performed in situ directly upon tissue sections (fixed and/or frozen) of patient tissue obtained from biopsies or resections, such that no RNA purification is necessary. Nucleic acid reagents such as those described in Section 5.1, and its subsections, may be used as probes and/or primers for such in situ procedures (Nuovo, G.J., 1992, PCR in situ hybridization: protocols and applications, Raven Press, NY).

PKD1 nucleotide sequences, either RNA or DNA, may, for example, be used in hybridization or amplification assays of biological samples to detect abnormalities of PKD1 expression; e.g., Southern or Northern analysis, single stranded conformational polymorphism (SSCP) analysis including in situ hybridization assays, alternatively, polymerase chain reaction analyses. Such analyses may reveal both quantitative abnormalities in the expression pattern of the PKD1 gene, and, if the PKD1 mutation is, for example, an extensive deletion, or the result of a chromosomal rearrangement, may reveal more qualitative aspects of the PKD1 abnormality.

Preferred diagnostic methods for the detection of PKD1 specific nucleic acid molecules may involve for example, contacting and incubating nucleic acids, derived from the target tissue being analyzed, with one or more labeled nucleic acid reagents as are described in Section 5.1, under conditions favorable for the specific annealing of these reagents to their complementary sequences within the target molecule. Preferably, the lengths of these nucleic acid reagents are at least 15 to 30 nucleotides. After incubation, all non-annealed nucleic acids are removed. The presence of nucleic acids from the target tissue which have hybridized, if any such molecules exist, is then detected. Using such a detection scheme, the target tissue nucleic acid may be immobilized, for example, to a solid support such as a membrane, or a plastic surface such as that on a microtiter plate or polystyrene beads. In this case, after incubation, non-annealed, labeled nucleic acid reagents of the type described in Section 5.1 and its subsections are easily removed. Detection of the remaining, annealed, labeled nucleic acid reagents is accomplished using standard techniques well-known to those in the art.

Alternative diagnostic methods for the detection of PKD1 specific nucleic acid molecules may involve their amplification, e.g., by PCR (the experimental embodiment set forth in Mullis, K.B., 1987, U.S. Patent No. 4,683,202),

ligase chain reaction (Barany, F., 1991, Proc. Natl. Acad. Sci. USA 88:189-193), self sustained sequence replication (Guatelli, J.C. et al., 1990, Proc. Natl. Acad. Sci. USA 87:1874-1878), transcriptional amplification system (Kwoh, D.Y et al., 1989, Proc. Natl. Acad. Sci. USA 86:1173-1177), Q-Beta Replicase (Lizardi, P.M. et al., 1988, Bio/Technology 6:1197), or any other RNA amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of RNA molecules if such molecules are present in very low numbers.

In one embodiment of such a detection scheme, a cDNA molecule is obtained from the target RNA molecule (e.g., by reverse transcription of the RNA molecule into cDNA). Tissues from which such RNA may be isolated include any tissue in which wild type PKD1 is known to be expressed, including, but not limited, to kidney tissue and lymphocyte tissue. A target sequence within the cDNA is then used as the template for a nucleic acid amplification reaction, such as a PCR amplification reaction, or the like. The nucleic acid reagents used as synthesis initiation reagents (e.g., primers) in the reverse transcription and nucleic acid amplification steps of this method are chosen from among the PKD1 nucleic acid reagents described in Section 5.1 and its subsections. The preferred lengths of such nucleic acid reagents are at least 15-30 nucleotides. For detection of the amplified product, the nucleic acid amplification may be performed using radioactively or non-radioactively labeled nucleotides. Alternatively, enough amplified product may be made such that the product may be visualized by standard ethidium bromide staining or by utilizing any other suitable nucleic acid staining method.

5.12.2. DETECTION OF PKD1 GENE PRODUCT AND PEPTIDES

Antibodies directed against wild type or mutant PKD1 gene product or peptides, which are discussed, above, in

Section 5.3, may also be used as ADPKD diagnostics, as described, for example, herein. Such diagnostic method, may be used to detect abnormalities in the level of PKD1 protein expression, or abnormalities in the location of the PKD1 tissue, cellular, or subcellular location of PKD1 protein. For example, in addition, differences in the size, electronegativity, or antigenicity of the mutant PKD1 protein relative to the normal PKD1 protein may also be detected.

Protein from the tissue to be analyzed may easily be isolated using techniques which are well known to those of skill in the art. The protein isolation methods employed herein may, for example, be such as those described in Harlow and Lane (Harlow, E. and Lane, D., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York), which is incorporated herein by reference in its entirety.

Preferred diagnostic methods for the detection of wild type or mutant PKD1 gene product or peptide molecules may involve, for example, immunoassays wherein PKD1 peptides are detected by their interaction with an anti-PKD1 specific peptide antibody.

For example, antibodies, or fragments of antibodies, such as those described, above, in Section 5.3, useful in the present invention may be used to quantitatively or qualitatively detect the presence of wild type or mutant PKD1 peptides. This can be accomplished, for example, by immunofluorescence techniques employing a fluorescently labeled antibody (see below) coupled with light microscopic, flow cytometric, or fluorimetric detection. Such techniques are especially preferred if PKD1 gene products or peptides are expressed on the cell surface.

The antibodies (or fragments thereof) useful in the present invention may, additionally, be employed histologically, as in immunofluorescence or immunoelectron microscopy, for *in situ* detection of PKD1 gene product or peptides. *In situ* detection may be accomplished by removing a histological specimen from a patient, and applying thereto

a labeled antibody of the present invention. The histological sample may be taken from a tissue suspected of exhibiting ADPKD. The antibody (or fragment) is preferably applied by overlaying the labeled antibody (or fragment) onto a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the PKD1 peptides, but also their distribution in the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

Immunoassays for wild type or mutant PKD1 gene product or peptides typically comprise incubating a biological sample, such as a biological fluid, a tissue extract, freshly harvested cells, or cells which have been incubated in tissue culture, in the presence of a detectably labeled antibody capable of identifying PKD1 peptides, and detecting the bound antibody by any of a number of techniques well-known in the art.

The biological sample may be brought in contact with and immobilized onto a solid phase support or carrier such as nitrocellulose, or other solid support which is capable of immobilizing cells, cell particles or soluble proteins. The support may then be washed with suitable buffers followed by treatment with the detectably labeled PKD1 specific antibody. The solid phase support may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on solid support may then be detected by conventional means.

By "solid phase support or carrier" is intended any support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually

any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of anti-wild type or mutant PKD1 peptide antibody may be determined according to well known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

One of the ways in which the PKD1 peptide-specific antibody can be detectably labeled is by linking the same to an enzyme and use in an enzyme immunoassay (EIA) (Voller, A., "The Enzyme Linked Immunosorbent Assay (ELISA)", Diagnostic Horizons 2:1-7, 1978) (Microbiological Associates Quarterly Publication, Walkersville, MD); Voller, A. *et al.*, *J. Clin. Pathol.* *31*:507-520 (1978); Butler, J.E., Meth. Enzymol. 73:482-523 (1981); Maggio, E. (ed.), ENZYME IMMUNOASSAY, CRC Press, Boca Raton, FL, 1980; Ishikawa, E. *et al.*, (eds.) ENZYME IMMUNOASSAY, Kgaku Shoin, Tokyo, 1981). The enzyme which is bound to the antibody will react with an appropriate substrate, preferably a chromogenic substrate, in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and

acetylcholinesterase. The detection can be accomplished by colorimetric methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection may be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments it is possible to detect PKD1 wild type or mutant peptides through the use of a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986, which is incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography.

It is also possible to label the antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol,

isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in, which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

6. EXAMPLE: DETERMINATION OF THE PKD1 INTERVAL VIA GENETIC POLYMORPHISM ANALYSIS

In the Working Example presented herein, genetic linkage studies are discussed which successfully reduced the potential PKD1 interval from approximately 750 kb to approximately 460 kb, thus substantially narrowing the genomic region in which the gene responsible for ADPKD lies.

6.1 MATERIALS AND METHODS

Sequencing techniques: Sequencing of cDNA clones and genomic clones was carried out using an Applied Biosystems ABI 373 automated sequencing machine according to the manufacturer's recommendations or by manual sequencing according to the method of Ausubel P. M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & Sons, New York, pp. 7.0.1 & ff.

Inserts from the cDNA phage clones were excised with EcoRI and ligated into the appropriate cloning sites in the polylinker of pBlueScript plasmid (Stratagene). Primers for sequencing of the plasmid clones were based on the known sequence of the polylinker. A second set of sequencing primers were based on the DNA sequences obtained from the first sequencing reactions. Sequences obtained using the second set of primers were used to design a third set of

primers and so on. Both strands of the double-stranded plasmids were sequenced.

PCR products were sequenced using the dsDNA cycle sequencing system of GIBCO-BRL (Gaithersburg, MD) according to the manufacturer's instructions. PCR product was purified, prior to sequencing, by passing the DNA through a Centricon column twice according to the manufacturer's instructions (Amicon, Beverly, MA, USA). 100-200ng of each purified PCR product was used as template in the sequencing reaction.

Genomic sequences were obtained from PCR products as well as from subclones from the cosmids. To ensure the correct locus sequence was obtained over the duplicated locus. Only cGGG10 and cDEB11 sequence was utilized when identifiying intron/exon boundaries.

DNA labelling: Double-stranded DNA probes were made by labelling DNA by the method of Feinberg and Vogelstein, 1983, Anal. Biochem. 132: 6-13. Primers were end-labelled with γ^{32}p-ATP using the method of Ausubel F. M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol- 1, Green Publishing Associates, Inc., and John Wiley & Sons, New York, pp. 4.8.2 &ff.

PCR conditions: Conditions for the PCR reactions were determined empirically for each reaction by analyzing an array of reaction conditions with the following variables: magnesium concentrations of 1mM, 2mM, 4mM; annealing temperature; extension time; primer concentration and primer concentration ratio.

The fixed conditions were:

1. extension at 72°C using Taq polymerase, 2.5u/100μl reaction volume;
2. denaturation at 95°C for 1 minute; and
3. annealing for 30 seconds.

Primer design: Primers were designed using the computer program "PRIMER".

Genetic linkage studies: Genetic linkage studies were carried out using computerized algorithms (Lathrop GM., et al., 1984, Proc. Natl. Acad. Sci. USA, 81:3443-3446; Lathrop GM and Lalouel J-M., 1984, Am. J. Hum. Genet. 36:460-465; Lathrop G.M., Lalouel J.-M., Julier C., Ott J., 1985, Am. J. Hum. Genet. 37:482-498).

Single-stranded conformational polymorhism analysis (SSCP): SSCP analysis to detect sequence polymorphisms was carried out according to the method of Orita et al, 1989, Genomics, 5:874-879. Primers were designed to amplify each exon (see figure 10 and Table 1, below). The 3' end of each primer was designed to lie ~20-50bp from the nearest intron/exon boundary so that mutations in the splice donor and acceptor sites could be detected.

Table 1: Primer Sequences from the PKD1 gene

Primer Name	Sequence (5'-3')	Sense/antisense
KG8-F9	CTGCCGGCCTGGTGTCG	sense
KG8-F11	AGGGTCCACACGGGCTCGG	sense
KG8-F23	CAGGGTGTCCGTGCGTGACTG	sense
KG8-F25	GTCCAGCACTCCTGGGGAGA	sense
KG8-F26	ACGCAAGGACAAGGGAGTAG	sense
KG8-F27	AGTGCCGCGGCCTCCTGAC	sense
KG8-F28	GCTGGCCTAGGCGGCTTCCA	sense
KG8-MF2	CACCCCACGGCTTTGCACT	sense
KG8-MF4	CCCAGGCAG CGAGGCTGTC	sense

KG8-RO2	ACACCAGGCCAACAGCGACTG	antisense
KG8-R9	ACAGCCACCAGGAGCAGGCTG A	antisense
KG8-R13	TGTAGCGCGTGAGCTCCAG	antisense
KG8-R23	CACCCCACCCTACCCCAG	antisense
KG8-R24	GGAGGCCACAGGTGAGGCT	antisense
KG8-R27	CGGAGGAGTGAGGTGGGCTCC	antisense
KG8-R28	AGCCATTGTGAGGACTCTCCC	antisense
NKG9-F2	AAGACCTGATCCAGCAGGTCC	sense
NKG9-F07	CAGCACGTCATCGTCAGG	sense
NKG9-R03	CTCCCAGCCACCTTGCTC	antisense
NKG9-R07	GCAGCTGTCGATGTCCAG	antisense
NKG9-RM2	TCTGTCCAACAAAGGCCTG	antisense

6.2 RESULTS

It was previously shown that the PKD1 gene maps, by genetic linkage, to the interval between the polymorphic genetic markers D16S259 (which lies on the telomeric side of PKD1) and D16S25 (which lies on the centromeric side of PKD1) (see Somlo et al., 1992, Genomics 13:152). The smallest interval between genetic markers, called the PKD1 interval was found to be approximately 750kb (see Germino et al., 1992, Genomics 13:144). The PKD1 interval was isolated as a series of forty overlapping cosmid and phage clones. The cloned DNA contained the entire PKD1 interval with the exception of two gaps of less than 10kb and less than 50kb (see FIG. 1; Germino et al., Genomics 13:144, 1992).

In the Example presented herein, in order to reduce the PKD1 interval still further, a systematic search for additional polymorphic markers was undertaken. Single-stranded DNA probes $(CA)_{8-15}$ were hybridized to the set of

clones from the PKD1 interval. The phage clone w5.2 (see FIG. 1) was found to hybridize to the probe and the sequence flanking the (CA)n (w5.2 repeat) was determined using phage DNA as a template. Primers for the polymerase chain reaction (PCR) were designed and used to detect polymorphism within the w5.2Ca repeat. The position of the w5.2Ca repeat is shown in FIG. 2. This w5.2Ca repeat was used in genetic linkage studies in 15 PKD1 families and found to lie proximal to the PKD1 locus. This experiment reduced the size of the PKD1 interval to approximately 460kb, as shown in FIG. 2.

7. EXAMPLE: IDENTIFICATION OF POTENTIAL PKD1 TRANSCRIPTS

In the Working Example presented herein, transcription units within the 460 kb PKD1 interval, (FIG.2) defined in Section 6, above, were identified. The interval was found to have a maximum of 27 transcriptional units (TU), which contained a total of approximately 300 kb.

7.1 Materials and Methods

cDNA library screening: cDNA libraries were prepared from several sources including EBV transformed lymphocytes, teratocarcinoma tissue, fetal kidney and HeLa cells. In addition a human adult kidney library was purchased from Clontech Inc. (San Diego, CA).

Total RNA from each tissue was prepared by the guanidinium chloride method. First strand cDNA synthesis was prepared using random six base oligonucleotides by the method of Zhou et al, Journal Biol. Chem., 267:12475 (1992). EcoRI sites within the cDNA were blocked by DNA methylase. The cDNA was flush-ended with T4 kinase and EcoRI linkers were added with DNA ligase. The cDNA was cleaved with EcoRI and ligated into either bacteriophage lambda-gt10 or lambda-ZAP (Stratagene). The phage were packaged with high-efficiency packaging extract (Stratagene). At least one million primary clones were plated. The library was amplified 100-fold and stored at 4° C.

At least 500,000 plaques of each library were screened with each cosmid clone at a density of 25,000 per 75mm diameter plate. Duplicate filter lifts were made of each plate (Ausubel, supra). The radiolabelled probes were incubated with an excess of unlabelled denatured human DNA and then added to the library filters in a sodium phosphate buffer at 65° C. for 16 hours. The filters were washed in 2xSSC at 65° C. for 1 hour and 0.1xSSC, 0.1xSDS at 65° C. for one hour. Kodak XAR-5 was exposed to the library filters for 4-16 hours. Duplicate positives were picked and replated at a density of approximately 100-500 per plate. Filter lifts of these secondary plates were made and hybridized as for the primary lifts; pure isolated plaques were obtained and inoculated into 50ml cultures and the phage DNA was purified.

Sequencing techniques: Techniques were as described in Section 6.1, above.

7.2 Results

To identify transcribed sequences within the PKD1 interval (FIG. 2), the cosmid and phage clones from the interval were hybridized to cDNA libraries made from a variety of human tissues including fetal and adult kidney, teratocarcinoma, adult liver, lymphoblast, HeLa, and adult brain. More than 100 hybridizing cDNA clones were identified. These clones were subcloned into pBlueScript plasmids and sequenced. The sequence data combined with hybridization data (between cDNA clone and genomic clone) allowed the cDNA clones to be assigned to a maximum of 27 transcription units, as described below.

Namely, hybridization between two cDNA clones was evidence that the clones are part of the same transcription units. Similarly, sequence identities of greater than 25bp between the cDNA clones were used as evidence that the clones were part of the same transcription unit.

Table 2, below, lists these units (a-z, aa) by the name of the longest clone.

Table 2

Putative Transcriptional Unit
Sequences Isolated From the PKD1 Region

CANDIDATE GENES IN THE PKD1 REGION

	Clone	Insert Size (kb)	cDNA Libraries	Motif
a.	20.7	2.1	cy, terat	
b.	SazD	2.7	cy	G-protein β subunit-like
c.	SazB	2.2	cy, terat	scERV from yeast
d.	Saz10	4.0	cy, lym	
e.	Saz13	1.5	cy, terat	tandem 120 amino-acid repeat: Z01 - family
f.	Saz20	5.5	cy, lym, terat	
g.	KG8	3.4	lym	
h.	NKG9	1.8	lym	
i.	NKG10	2.8	lym	
j.	NKG11	2.4	lym	
k.	Nik4	0.9	kid	
l.	Nik7	2.3	lym, terat	rab gene motif
m.	KG3	3.8	terat, cy	G-protein β subunit-like
n.	Nik9	2.2	cy	ankyrin repeat
o.	KG4	0.6	kid	
p.	KM17	1.6	terat, cy	G-protein β subunit-like
q.	Nik10	1.6	lym	
r.	KG5	2.6	cy	zinc-finger protein
s.	KG1	1.1	kid	DNase
t.	KG6	3.4	kid, cy, lym	human homolog of mouse RNSP1 gene
u.	Nik3	3.2	terat, lym, cy	*
v.	Nik2	3.4	terat, lym, cy	*
w.	Nik1	0.8	kid	*
x.	Nik8	1.6	lym	*
y.	KG17	2.2	lym	
z.	AJ1	1.4	cy	cyclin-F homolog
aa.	MAR1	2.0	kid	MDR-like

* u, v, w, x are part of an 8kb transcriptional unit (nik 823) which produces a MDR-like channel. MAR1 is another member of the gene family. ATP-dependent transporter cyclin proton-channel of vacuolar proton ATPase
cDNA library from which the clone was obtained: cy=cyst; terat=teratocarcinoma; lym=lymphoblast; kid=kidney

Thus, these 27 transcription units were considered by virtue of their genomic localization to be candidate genes for PKD1. The total transcribed cDNA in the 27 transcription units equalled about 60kb.

The sequence of each clone was compared with sequences deposited in the public databases Genbank, EMBL, and SwissProt. Several of the cDNA clones contained sequences predicted to code for known protein motifs. Because so little was known of the molecular basis of ADPKD none of the candidate genes could be ruled out by virtue of sequence motifs.

8. PKD1 INTERVAL NORTHERN ANALYSIS

In the Working Example presented herein, an analysis of the transcriptional expression patterns of the TUs described, above, in Section 7, was conducted.

8.1 MATERIALS AND METHODS

Northern blot analysis: Poly A+ RNA (2μg) from heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas was hybridized with radio-labelled cDNA probes from the TUs within the PKD1 interval, under standard conditions.

8.2 RESULTS

Inserts from the cDNA clones of the TUs described in Section 7,and listed in Table 2, above, were used to probe Northern blots containing total RNA and polyA-enriched RNA from normal human organs and from between 8 and 10 kidneys removed from patients with ADPKD.

The expression profile was compared with the pattern of pathology in ADPKD to determine a priority for further characterization. The Northern analysis demonstrated that 26 of the TUs in the PKD1 interval were expressed in kidney, with the exception of Nik9. Nik9 mRNA was found to be abundant in human brain but expressed at very low level in fetal and adult human kidney. These data, therefore, indicated that Nik9 is not the PKD1 gene. No consistent

differences were observed between normal and ADPKD kidneys for any transcript.

9. EXAMPLE: PKD1 INTERVAL MUTATION SCREENS

A systematic search was undertaken to detect mutations in ADPKD patients in the transcribed regions listed in Table 2. The mutation screen used several independent techniques. Southern blot analysis of patient DNA digested with at least three different restriction endonucleases was performed. Several differences between the restriction patterns were detected but none was found only in patients with ADPKD. Single-stranded conformational polymorphism analysis was carried out using cDNA isolated from patient transformed lymphocytes as a template. A large number of allelic differences was found but none were found to alter the deduced product of transcription. Sequence analysis of the KG5 cDNA was carried out in seven ADPKD patients and one normal. The deduced coding region of 2.6kb was sequenced using cDNA, made by reverse transcription from patient transformed lymphocyte mRNA, as a template. The cDNA was amplified by PCR in a series of overlapping sections and the PCR products were sequenced. No sequence differences were detected between patients and normal individuals. In this way more than 80% of the coding DNA in the transcription units was scanned and no mutations were found in PKD1 patients. These experiments excluded the scanned segments of the transcription units with a likelihood of 95% based on the reasonable assumption that no ADPKD mutation accounts for >70% of all ADPKD cases.

Thus, the following transcription units were excluded: sazB, sazD saz13, KG3, KG5, KGI, saz20, KM17, Nik1, Nik2, Nik3, Nik8, KG17, Nik7, MAR1. These excluded transcripts represent >80% of the combined identified coding sequences in the PKD1 region.

It has previously been noted that <u>de novo</u> mutation to ADPKD accounts for at least 1% of cases. Two mechanisms have been shown to account for the vast majority of new mutation

rates of this order. First, the coding region may be large. Duchenne muscular dystrophy (DMD) provides an example of this situation: the dystrophin gene which is mutated in DMD has a transcript of approximately 14kb. About 30% of DMD cases arise by de novo mutation. The second mechanism that may account for a high new mutation rate is the presence of an unstable repetitive element. Unstable trinucleotide repeats in which the repeat sequence contains >50% C and G have been shown to cause the fragile X syndrome, Huntington's disease and myotonic dystrophy. In two of these diseases, high mutation rates or the appearance of progressively more severe disease in successive generations (anticipation) have been documented.

A systematic search for trinucleotide repeats in the PKD1 interval was undertaken. Single-stranded probes (15-25 nucleotides) containing all possible combinations of trinucleotide repeats were synthesized, radiolabelled and hybridized to Southern blots containing the complete set of clones comprising the PKD1 interval. The hybridization and washing conditions were adjusted to allow detection of all perfect repeats of 15 nucleotides or more. Eight separate banks of trinucleotide repeats within the PKD1 interval were found. Primers were designed so that the trinucleotide repeat arrays could be amplified by PCR and size-fractionated on polyacrylamide gels. No differences were found between ADPKD patients and controls.

Additionally, two other screening methods were attempted for the identification of trinucleotide expansions in the PKD1 interval. Southern blots of DNA from normal and affected individuals was probed with inserts containing the repeats. This revealed no polymporphisms. Further, multiply restricted DNA samples (Rsa/Sau3A/Hinf1) samples were probed with trinucleotide repeat oligonucleotides. Though myotonic dystrophy and fragile-X mental retardation patients could be identified via such methods, it was not possible to identify any common pattern in ADPKD patients.

The cDNA clones Nik1, Nik2, Nik3, and Nik8 were found to hybridize to an 8kb transcript present in kidney. These clones were assumed to be part of the same transcript. PCR product that bridged the three gaps in sequence between the four clones were obtained using primers based on sequences within the four cDNA clones. In this way approximately 8kb of the transcribed DNA sequence of the gene represented by Nik1, Nik2, Nik3, and Nik8 was obtained. Because the coding region is large the gene was expected to have a high spontaneous mutation rate and therefore to be a good candidate for the PKD1 gene. A detailed exon-by-exon search of the gene, however, revealed no evidence of mutations in ADPKD patients. This left only one TU within the region which was considered large enough to be a reasonable candidate for the PKD1 gene. The characterization of clones and sequences within this TU, part of the putative PKD1 gene, is described, below, in the Working Examples presented in Sections 10 and 11.

10. EXAMPLE: SSCP Analysis of ADPKD Patients

In the Working Example presented herein, an SSCP analysis of genomic DNA amplified from DNA derived from normal and ADPKD patients was conducted which identified ADPKD-specific allelic differences which map to the single gene of the PKD1 interval which was described, above, in the Working Example presented in Section 10.

10.1 Materials and Methods

SSCP Analysis: Single-Stranded Conformational Analysis (SSCP) was performed as follows: 50ng of genomic DNA was amplified by PCR under standard conditions in a reaction volume of 20 μl. Ten microliters of the amplified product was added to 90 μl of formamide buffer, heated at 97°C for 4-5 minutes, and cooled on ice. Four microliters of the reaction mixture was loaded on a polyacrylamide gel (10%, 50:1 acrylamide:bisacrylamide) containing 10% glycerol. The gel was run at 4°C for 12 hours with 10W power in 0.5 X TBE

buffer. The gel was dried and exposed to a Molecular Dynamic Phosphor-Imager screen for 4 to 16 hours.

Intron/Exon Mapping: Primers produced from cDNA clones were used to PCR amplify genomic DNA sequences. Amplified products were sequenced, using standard methods. Those sequences which differed from the cDNA sequences indicated intron sequences.

PCR Amplification: Procedures for amplification were as described, above, in Section 6.1.

10.2 Results

Because the large size of the putative KG8/NKG9/NKG10/NKG11 transcript makes it a likely site for mutation, the intron/exon structure of part of the gene represented by KG8 and NKG9 was determined so that an exon-by-exon search for mutations could be conducted. The exon/intron structure analysis allowed PCR primers to be designed for the amplification of several exons of the PKD1 gene.

These primers were used to PCR-amplify genomic DNA and to perform SSCP of ADPKD patients and normal individuals. In two ADPKD patients SSCP patterns were observed that showed allelic differences. Both patients were heterozygous for an SSCP variant that was not seen in a large number of normals from the normal population (Fig 3A-3B). In samples from these two individuals, 4 bands are visible, instead of the 2 single-strand bands seen in samples from normal individuals. The 4 bands are of equal intensity and are presumed to comprise two allelic sense strand and two allelic antisense strands.

Thus, the results discussed in this Example, coupled with the analyses reported, above, in the Examples presented in Sections 6 through 9 provide positive correlative evidence that the gene corresponding to the putative transcription

unit of which the clones KG8, NKG9, NKG10 and NKG11 are believed to be a part, is the PKD1 gene.

11. EXAMPLE: MOLECULAR CHARACTERIZATION OF THE PKD1 GENE

In this Example, the complex structure of the PKD1 gene and gene product is described. Included herein is a description of the PKD1 gene structure, the nucleotide sequence of the entire coding region of the PKD1 transcript, as well as the amino acid sequence and domain structure of the PKD1 gene product. This description not only represents the first elucidation of the entire PKD1 coding sequence, but additionally also corrects errors in the portion of the PKD1 coding region which had previously been reported. Also, a ADPKD-1 causing mutation within the PKD1 gene which results in a frameshift is identified. Further, the strategy utilized to characterize this extensive and difficult nucleic acid region is summarized.

A portion of the nucleotide sequence corresponding, in large part, to the 3' end of the PKD1 gene had recently been reported (European Polysystic Kidney Disease Consortium [hereinafter abbreviated EPKDC], 1994, Cell 77:881-894). Specifically, the terminal 5.6 kb of the PKD1 transcript were studied and an open reading frame of 4.8 kb was reported. The peptide this putative open reading frame encodes, which would correspond to the carboxy terminal portion of the PKD1 protein, did not reveal any homologies to known proteins and, if this derived amino acid sequence was, in fact, part of the PKD1 protein, its sequence did not suggest a function for the PKD1 gene product.

For this lack of revealing information, in addition to the fact that only a small percentage of ADPKD-causing mutations appear to reside within the 3' end of the PKD1 gene, the characterization of the 5' end of the gene and a more complete analysis of the PKD1 gene and gene product were greatly needed.

As acknowledged by the EPKDC (EPKDC, 1994, Cell 77:881-894), however, the elucidation of the complete PKD1 coding

sequence presents major problems. Unlike the 3' end of the PKD1 gene, the 5' two-thirds of the gene appear to be duplicated several times at other genomic positions. Further, at least some of these duplications are transcribed. Thus, great difficulties arise when attempting to distinguish sequence derived from the authentic PKD1 locus apart from sequence obtained from the duplicated PKD1-like loci.

11.1. MATERIALS AND METHODS

11.1.1. GENOMIC CLONES

The human P1 phage named PKD 1521 was isolated from a human P1 library using primers from the adjacent TSC2 gene. The first screen utilized primers F33tcttctccaacttcacggctg, R32aaccagccaggtttggtcct, followed by F38caagtccagctcctctccc, R40gctctttaaggcgtccctc and ultimately screened with primers in the KG8 gene (F9/R5) see page 68 for KG8-R5 5' primer, while KG8-R5 5' gcgctttgcagacggtaggog 3'. The cosmid cGGG10 has been previously described (Germino, G.G., Weinstat-Saslow, D., Himmelbauer, H., Gillespie G.A.J., Somlo, S., Wirth, B., Barton, N., Harris, K.L., Frischauf, A.M. and Reeders, S.T. (1992) Genomics, 13:144-151). The cosmid cGGG10 was mapped using various restriction enzymes as described by the manufacturers. A random library of the cosmid was constructed by cloning sheared DNA fragments into the SmaI site of pUC 19. Initial sequence assembly for the cosmid cGGG10 was performed on forward and reverse sequences of approximately 1000 random cloned fragments and a preliminary map was constructed using the restriction map of the cosmid. Directed subclones of cGGG10 were made in the plasmid pBluescript (Stratagene) in order to create sequencing islands specific physical locations. These large subclones from cGGG10 were then restricted with more frequent cutter enzymes and cloned into M13mp19 and mp18. In addition, if gaps were found in cloned regions, directed sequencing was performed from the flanking regions, to join the anchored contigs. A contig of 34.3 Kb was constructed, with two gaps in what appear to be highly repetitive regions

with no identifiable coding sequence. cDEB11 was has been described previously (Germino, G.G., Weinstat-Saslow, D., Himmelbauer, H., Gillespie G.A.J., Somlo, S., Wirth, B., Barton, N., Harris, K.L., Frischauf, A.M. and Reeders, S.T. (1992) Genomics, 13:144-151). A random library was constructed with sheared cDEB11 DNA and cloned into the SmaI site of pUC19. This cosmid was sequenced to obtain at least 2-fold coverage.

The sequencing was done by cycle sequencing and run on ABI machines following the manufacturer's instructions with modifications as described below. Because of the difficulty of sequencing certain regions, the standard chemistry of sequencing used withthe ABI machines had to be modified. Both dye terminator and dye primer sequence were used when appropriate with sequencing different regions. Different polymerases and different melting and polymerization conditions were also used in order to optimize the quality of the sequence. When sequencing across the CpG island at the 5' end of the PKD1 gene, the best sequencing results were obtained when adding 5% DMSO to the polymerization step and sequencing single-stranded templates.

11.1.2. <u>cDNA LIBRARY SCREENING</u>

The first cDNA used to screen libraries was KG8, which maps to the unique region of the PKD1 locus and was recovered from an adult lymphocyte libary. In order to complete the rest of the PKD1 transcript, fourteen new cDNAs were sequenced to completion, four cDNAs were partially sequenced and an additional 20 cDNAs were mapped against cGGG10. Additional data was obtained from RT-PCR products of the renal cell carcinoma cell line SW839 (ATCC).

Overlapping partial cDNAs described below were isolated from lymphocyte and fetal kidney libraries. In this way, a 14 kb transcript was assembled starting from the 3' until the CpG island was reached. It is assumed that the 5'end of the PKD1 trancript has been located. No other clones further upstream were recovered upon further screening those cDNA

libraries that had provided the majority of the cDNAs which were used to assemble the full length PKD1 cDNA.

The cDNAs FK7 and FK11 were recovered from a fetal (gestation age of 14-16 weeks) kidney cDNA library using KG8 cDNA as a probe. This library was constructed with the Superscript Lambda System from (Gibco/BRL), using oligo d(T) primed cDNA. FK7 and FK11 were recovered as SAlI inserts. The cDNAs designated BK156, BK194, UN49 and UN52 were recovered from a lymphocyte cell library and pulled by using FK7 as a probe. UN34 was recovered from the same library by hybridizing with a ScaI-SaII 5'end probe of FK7. UN53, UN54 and UN59 were recovered from the same lymphocyte library (M. Owen laboratory, ICRF; Dunne, PhD thesis, 1994) by double screening clones that were both negative when screening with an FK7 probe and positive when screening with BK156 and UN52. The cDNA NKG11 was recovered from a lymphocyte library screened with cGGG10 and was described previously (Germino, G.G., Weinstat-Saslow, D., Himmelbauer, H., Gillespie G.A.J., Somlo, S., Wirth, B., Barton, N., Harris, K.L., Frischauf, A.M. and Reeders, S.T. (1992) Genomics, 13:144-151).). The cDNA named Fhkb21 was obtained from a Clonetech fetal kidney library using BK156 as a probe. MSK3 was obtained by probing an adult kidney library (Clonetech) with a probe from 5'end of KG8. MSK4 was obtained by nested RT-PCR from primers spanning from exons 7-8 to exons 13-14, followed by second round of PCR with internal primers in exon 8 and exon 13.

11.1.3. cDNA SEQUENCING

The cDNAs were sequenced to 5-fold coverage by primer walking and/or subloning small fragments into M13 or pBluescript. All cDNA sequences were compared to the cGGG10 cosmid sequence to assess whether they were from the correct locus and to determine intron/exon boundaries. Discrepancies were resequenced to determine whether the differences were genuine. Some of the cDNAs described above were clearly different from the genomic sequence, suggesting that these cDNAs were encoded by another locus.

MSK3, FK7 and FK11 were obtained using a PKD1-specific probe (KG8) were found to be 100% identical to genomic sequence. The cDNA and UN49, which showed 99% identity, is possibly PKD1-specific. BK241, BK194, UN52, UN53, UN54 and UN59, BK156, Fhkb21 and NKG11 were 96-98% homologous to the cGGG10 defined exon sequence, and thus were assumed to have originated fromt the duplicated loci. In general, differences between genomic cDNA were nucleotide differences scattered through out the cDNA sequence. One exception is BK194, which has an extra CAG at position 1863 of the previously published partial sequence and arose from alternative splicing of exon 33. Another exception is BK241 that has an insertion of the following sequence in a tandem repeat of TTATCAATACTCTGGCTGACCATCGTCA at position 1840 of the previously published sequence (European PKD1 Consortium). This sequence was not included in the authentic, full-length PKD1 cDNA because it arose from the duplicated loci would produce a frame shift in the ccoding region of the PKD1 transcript. Except for BK241, cDNAs in the UN and BK series that overlap with each other are more identical to themselves than to the genomic sequence.

All sequence assembly was performed using the Staden package XBAP (Dear, S. and Staden R. (1991). Nucleic Acid Res. 19:3907-3911.)

11.1.4. <u>PROTEIN HOMOLOGY SEARCHES</u>

The PKD1 derived amino acid sequence was subjected to various sequence analysis methods (Koonin, E.V., Bork, P. and Sanders, C. (1994) Yeast chromosome III: new gene functions. EMBO 13:493-503). For identifying homologues, initial (SWISSPROT, PIR, GENPEPT, TREMBL, EMBL, GENBANK, NRDB) database searches were performed using the blast series of programs (Altschul, S.F. and Lipman, D.J., 1990, Proc. Natl. Acad. Sci. USA <u>87</u>:5509-5513) by applying filter for compositionally biased regions. (Altschul, S.F. et al., 1994, Nat. Genet. 6:119-129). By default, the BLOSUM62 amino acid exchange matrix was used (Henikoff, S. and Henikoff J.G.

(1993). Proteins 17:97-61). In order to reveal additional candidate preoteins that might be homologous to PKD1, the BLOSUM45 and PAM240 matrices were also applied. Putative homolgoues with a blast p-value below 0.1 were studied in detail. Multiple alignments of the candidate domains were carried out using CLUSTALW (Thompson, J.D., Higgins, D.G. and Gibson, T. (1994). Nucleic Acid Res. 22:4673-4680) and pattern (Rohde, K. and Bork, P. (1993). Comput. Appl. Biosci. 9:183-189), motifs and profiles (Grisbskov, M., McLachlan, A.D. and Eisenberg, D. (1987) Proc. Natl. Acad. Sci. USA 84:4355-4358 were derived. With all these constructs interactive database searches were performed. Results of these database searches were used for improving the multiple alignments that were then used for the next round of database searches. The final multiple alignment containing all retrieved members of a module family was then used as input for the secondary structure predictions (Rost, B. and Sander, C. (1994). Proteins 19:55-872).

11.1.5. SSCP ANALYSIS

Single-Stranded Conformational Analysis (SSCP) was performed as follows: 50ng of total genomic DNA was amplified by PCR. In addition to the genomic DNA, each PCR reaction contained 1 picomole of each primer (see below), 0.1 μl ^{32}P dATP (Amersham), 0.2 μl in AmpliTaq (Pharmacia), in PCR buffer with a final Mg^{2+} of 1.5 mM in a final volume of 20 μl. The amplification was performed for 25 cycles, each consisting of 94° C. for 30 seconds, 60° C. for 30 seconds, and 72° for 60 seconds.

Intronic primers F25 and Mill-1R were utilized for the initial SSCP evaluation. The fragment amplified with these primers overlaps with the 5′ end of KG8. Subsequently, the primers F31 and R35 were used to amplify the fragment used to sequence the PKD1 mutation.

Primers: F25 (5' TCGGGGCAGCCTCTTCCTG 3');
Mill-1R (5' TACAGGGAGGGGCTAGGG 3');
F31 (5' TGCAACTGCCTCCTGGAGG 3')
R35 (5' GGTCTGTCTCTGCTTCCC 3')

One microliter of each sample was diluted into loading dye (95% formamide, 20 mM NaOH, 1 mM EDTA, xylene cyanol, bromophenol blue) denatured at 98°C for 5 minutes, cooled on ice and loaded onto a 10% (50:1 acrylamide:bisacrylamide) polyacrylamide gel containing 10% glycerol. The gel was run at 4°C., 50 watts, for 3 hours. Exposure was overnight on phosphoimager plates.

Amplified DNA from the one individual with a variant pattern was then reamplified using KG8-F31 and KG8-R35 primers and the above-described PCR conditions. Both reamplified strands were then sequenced using standard procedures for cycle sequencing of PCR products. ^{32}P-dCTP incorporation was used.

11.2 RESULTS

A series of overlapping cosmid clones spanning the predicted PKD1 region has been described (Germino, G.G., Weinstat-Saslow, D., Himmelbauer, H., Gillespie G.A.J., Somlo, S., Wirth, B., Barton, N., Harris, K.L., Frischauf, A.M. and Reeders, S.T. (1992). Genomics, 13:144-151). The integrity of the cosmid contig was confirmed by long-range restriction mapping and genetic linkage analysis of polymorphic sequences derived from the cosmids. Three cosmids (cGGG1, cGGG10 and cDEB11, from centromere to telomere) form a contig that includes the 3' end of the adjacent gene, TSC2, (cDEB11) and spans over 80 kilobases centromeric. At the proximal end of cGGg10, there is a CpG island represented by the Not I site, N54T (FIG. 1A).

In order to identify transcripts from the region, the cosmid clones were hybridized to a set of five cDNA libraries. KG8, a cDNA corresponding to the distal 3.2kb of the PKD1 sequence (which is located on cosmid cDEB11), was mapped using a panel of somatic cell hybrids, and found to hybridize to a single locus on chromosome 16p13. Sequence

analysis confirmed that KG8 contains the polyadenylated 3'end of a gene and has an open reading frame (ORF) of 2100 bp and a 1068 bp 3' untranslated region. KG8 was also found to contain a polymorphic (CA) microsatellite repeat. Analysis of this repeat in a large number of PKD1 kindreds revealed no recombination (supra.).

To obtain clones extending 5' of KG8, the cosmids cGGG10 and cDEB11 were hybridized to different cDNA libraries. When some of the positive clones obtained from these screens were analyzed using somatic cell hybrid panels, they were found to hybridize strongly to several loci on chromosome 16 in addition to the PKD1 region. The restriction maps of the hybridizing loci were so similar that it was concluded that a series of recent duplications of part of the PKD1 gene had occurred (excluding the PKD1 region from which the KG8 cDNA is derived) which had given rise to several PKD1-like genomic segments. This sequence duplication had been reported by the European PKD1 Consortium (EPKDC, 1994, Cell 77:881-894).

Preliminary sequence analysis of the cDNA clones revealed that the PKD1 and PKD1-like loci give rise to two or more transcripts sharing 95-98% sequence identity. Because of the high degree of similarity between PKD1 and PKD1-like transcripts, therefore, it was not possible to determine the correct full-length PKD1 cDNA sequence by simply assembling overlapping partial cDNA clones.

To begin to determine the sequence of the authentic PKD1 transcript, therefore, it was concluded that genomic PKD1 sequence should be compared to that of the PKD1 specific and PKD1-like cDNAs homologous to the genomic sequence. To that end, the entire cGGG10 cosmid and PKD1 exon-containing parts of the cDEB11 cosmid were sequenced, as described below.

11.2.1 SEQUENCE OF THE GENOMIC REGION OF THE PKD1 LOCUS

The duplicated portion of the PKD1 gene is largely contained within the cosmid cGGG10. Prior to sequencing cGGG10, the integrity of the clone was established in several ways. First, the restriction map of cGGG10 was compared with

map of the genomic DNA from the PKD1 region. Second, restriction maps of the overlapping portions of cGGG1 and cDEB11 were compared with cGGG10. Third, sequences derived from cGGG10 and overlapping portions of cDEB11 showed 100% similarity. Finally, a P1 phage, PKD1521, was obtained by screening a genomic P1 library with primers from the TSC2 gene, which maps near the PKD1 gene. No sequence differences were obtained between PKD 1521 and cGGG10.

It was necessary to pursue several approaches to obtain the sequence of cGGG10 (see Section 11.1, above). Briefly, due to the difficulty of sequence certain regions, modifications to standard automated sequencing chemistries had to be made. Both dye terminator and dye primer sequence was used, when appropriate, with several different regions. Further, different polymerases and different meltng and polymerization conditions were necessary to optimize the quality of the nucleotide sequence. When sequencing across the CpG island at the 5′ end of the PKD1 gene, in addition to modifying the polymerization step, single-stranded templates were used.

A final ten fold redundancy was achieved for the cGGG10 cosmid in order to be able to accurately compare the genomic sequence with that of the PKD1 specific and PKD1-like cDNAs homologous to this cosmid. The cGGG10 sequences were assembled into three contigs of 8 kb, 23 kb and 4.4 kb, separated by 1 kb and 2.2 kb gaps. A two-fold redundancy was obtained for the cDEB11 cosmid, whose sequence was compared to PKD1 locus specific cDNAs in order to obtain intron/exon boundaries of the unique 3′end of the PKD1 gene.

11.2.2. PKD1 and PKD1-LIKE cDNAs

In order to identify putative coding regions and intron/exon boundaries, genomic and cDNA sequences were compared. cDNA clones had been identified in two ways. First, fragments of cosmids cGGG10 and cDEB were hybridized to five cDNA libraries. Second, each cDNA clone was hybridized to fetal kidney and lymphocyte cDNA libraries to

obtain overlapping clones with which to extend the sequence (FIG. 1B).

When the sequences of overlapping cDNAs were assembled, a PKD1 trancript length of 14.2 kb was obtained. The predominant transcript detected by Northern analysis using the unique sequence KG8 probe is approximately 14 kb, suggesting that the cDNA clones represent the full-length of the PKD1 trancript.

Restriction and sequence analyses indicate that a CpG island overlaps the 5' end of the sequence. CpG islands hae been found to mark the 5' ends of many genes. Further, the most 5' cDNA clones (UN53, UN54 and UN59) each have identical 5' ends, providing additional evidence that no upstream PKD1 exons were missed (see Section 11.1, above).

The multiple cDNAs used to assemble the PKD1 trancript along with the genomic sequence are shown in FIGS. 1A and 1B. By comaring the sequences of overlapping cDNAs and analyzing the degree of homology between the different cDNAs and genomic sequence, it was possible to distinguish cDNAs encoded by the authentic PKD1 locus frm those encoded y the homologous loci (see Section 11.1, above). The full length PKD1 trancript constructed from these exons produces a large continuous open reading frame of 12,902 bp.

Significant sequence heterogeneity ws observed in these cDNAs, suggesting that some level of alternative splicing of the primary PKD1 transcript occurs. For this reason, it was sought to isolate a minimum of two cDNAs containing each exon, in order to increase the probability that all exons contributing to the PKD1 transcript were detected. Formally, however, it remains possible that there exist PKD1 transcripts which ccontain exons that are not present in the cDNA clones samples here.

Exon 17 was found in two cDNA clones (UN34 and BK156) and in the cosmid sequence, but the exon was not incorporated into the final PKD1 transcript. This is due to a number of reasons. First, the cDNA clones in which this exon is found differed from the cosmid and are likely to represent PKD1-

like genes, rather than the authentic PKD1 gene (see Section 11.1, above). Second, this exon is not found in FK1, a cDNA which was cloned using a PKD1-specific probe (KG8). Finally, when included in the full-length cDNA, this exon introduces a stop codon (743 nucleotides downstream of exon 17) that would producce a truncated protein of 2651 amino acid residues. Further studies are needed to assess whether this exon may be used in diffferent splice combinations in locus-specific trancripts. An ADPKD patient with a heterozygous mutation which introduces a stop codon at position 10,601 of the PKD1 open reading frame. Other mutations tha truncate the PKD1 protein have also been reported by the European PKD1 Consortium. Therefore, it is unlikely that transcripts which include exon 17 are predomiant forms in the kidney.

11.2.3. SEQUENCE ANALYSIS OF THE PREDICTED PKD1 PROTEIN

The assembly of 46 PKD1 exons yields a predicted transcript is 14.2 kb in length with 228 bp nucleotides of putative 5′ untranslated and 790 nucleotides of 3′ untranslated sequence. The authentic PKD1 transcript differs from the reported 3′ PKD1 sequence (EPKDC, 1994, Cell 77:881-894) due to the presence of two extra cytosines at position 12873 of the PKD1 open reading frame (corresponding to PBP position 4563). This frameshift yielded an erroneous carboxy PKD1 derived amino acid sequence which contained almost 80 additional amino acid residues. The presence of the two extra cytosines as confirmed with the cosmid sequence derived from cDEB11.

The PKD1 protein derived from the assembled PKD1 transcript is 4304 amino acids in length, with a predicted molecular weight of 462 kilodaltons. The nucleotide sequence encompassing the Met-1 codon is CTAACGATGC, which represents an uncommon translation start site (Kozak, M. (1984). Nucleic Acids Res. 12:857-872). This methionine was determined to be the putative PKD1 translation start site because it is preceded by an in-frame stop codon 63 bases upstream. Furthermore, the PKD1 coding region begins with a

23 amino acid region which exhibits many of the properties of a signal peptide and corresponding cleavage site (von Hejne, G. (1986). Nucleic Acids Res. 14:4683-4690. Welling, L.W. Grantham, J.J. (1972). J. Clin. Invest. 51:1063-1075).

In addition to the signal sequence, the identification of five domains that have been identified in other proteins and a newly discovered domain strongly suggests the extracellular location of at least the N-terminal half of the protein. Immediately downstream of the signal sequence there are two leucine-rich repeats (LRRs) (Figure 7A-7B). These LRRs are flanked on both sides by a cysteine rich regions which have homology to the flanking regions of a subset of other LRRS. LRRs occur in numerous proteins and have been shown to be involved in diverse forms of protein-protein interactions. The number of LRR within the respective proteins varies between 2 and 29 (Kobe B. and Deisenhofer J., 1994, Trends. Biochem. Sci. 19:415-421). Adhesive platelet glycoproteins form the largest group in the LRR superfamily (Kobe B. and Deisenhofer J., 1994, Trends. Biochem. Sci. 19:415-421). The structure of the array of 15 LRRs in porcine ribonuclease inhibitor (RI) has recently been crystallized (Kobe B. and Deisenhofer J., 1995, Nature 374:183-186); the LRRs of the RI protein form a horseshoe-like structure that surrounds RNase A (Kobe B. and Deisenhofer J., 1995, Nature 374:183-186). It has been suggested that proteins containing only a few LRR, like the PKD1 protein, interact with other proteins via the LRRs in order to form the horseshoe-like superstructure for protein-binding (Kobe B. and Deisenhofer J., 1994, <u>supra.</u>.

Although LRRs occur in various locations in different proteins, the additional flanking cysteine-rich disulfide bridge-containing domains, define a subgroup of extracellular proteins (Kobe B. and Deisenhofer J., 1994, <u>supra</u>. Only a few proteins have been sequenced so far that contain both, the distinct N-terminal and C-terminal flanking cysteine-rich domains (Figures 7A-7B and 8). Among this group are toll, slit, trk, trkB and trkC, which are all involved in cellular

signal transduction. For example, the Drosophila toll protein is suspected to be involved in either adhesion or signaling required to mediate developmental events such as dorsal-ventral patterning (Hashimoto, C., Hudson, K.L., and Anderson, K.V., 1988, Cell 52:269-279). The Drosophila slit protein is thought to possible mediate interactions between growing axons and the surrounding matrix (Rothberg, J.M., Jacobs, J.R., Goodman, C.S., and Artavanis-Tsakonas, S., 1990, Genes and Dev. 4:2169-2187). In vertebrates, these domains are found in the trk family of tyrosine kinase receptors; these proteins may relay cell or matrix adhesive events to the cytoplasm via a small carboxy terminal kinase domain (Schneider, R., Schweider, M., 1991, Oncogene 6:1807-11). it is interesting to note that all of the proteins with these cysteine-rich domains are involved in extracellular function, many of which relate to cell adhesion. For example, the platelet glycoproteins I and V help mediate the adhesion of platelets to sites of vascular injury. The 5T4 oncofetal trophoblast glycoprotein appears to be highly expressed in metastatic tumors.

The PKD1 protein also contains a single domain with homologies to C-type (calcium-dependent) lectin proteins (Figures 7A-7B and 8). These domains are believed to be involved in the extracellular binding of carbohydrate residues for diverse purposes, including internalization of glycosylated-enzyme (asialoglycoprotein receptors), participation in extracellular matrix (versican) and cell adhesion (selectins). The classification of C-type lectins has been based on exon organization and the nature and arrangement of domains within the protein. For example, class I (extracellular proteoglycans) and class II (type II transmembrane receptors) all have three exons encoding for the carbohydrate recognition domain (CRD); where as in classes III (collectins) and IV (LEC-CAMS) the domains are encoded by a single exon. The CRD in PKD1 C-type lectin domain does not fit into the above classification because it has a novel combination of protein domains and because it is

encoded by two exons (exons 5 and 6, Figure 6A-6P). Previous analysis has failed to establish a correlation between the type of carbohydrate bound to each C-type lectin and the primary structure of its CRD.

Exon 10 encodes a LDL-A module (from amino acids 642-672, Figures 7A-7B), a cysteine-rich domain of about 40 amino acids in length. This module was originally identified in the LDL-receptor but it is also present extracellular portions of many other proteins, often in tandem arrays (Figure 7A-7B). Because of their hydrophobic nature, these domains have been implicated as ligand-binding regions in LDL receptor-related protein. Other proteins, like the PKD1 protein, that contain a single or nontandem LDL-A, include the complement proteins (DiScipio, R.G., Gehring, M.R., Podack, E.R., Kan, C.C. Hugli, T.E., and Fey., G.H., 1984, Proc. Natl. Acad. Sci. USA 81:7298-7302), calf enterokinase (Kitamoto, Y., Yan, X.W., McCourt, D.W. and Sadler, J.E., 1994, Proc. Natl. Acad. Sci. USA 91:7588-7592) and a sarcoma virus adhesion protein.

In addition to extracellular protein modules that have been recognized previously, the PKD1 protein a novel domain of approximately 70 amino acids in length, present in 14 copies (Figures 7A-7B and 8). The first one is encoded by exon 5 between the LRRs and the C-type lectin module. The other PKD domains are consecutively placed starting at amino acid 1100 and ending at amino acid 2331 and contained in exons 13, 14, and 15. Profile and motif searches (see Section 11.1, above) identified several other extracellular proteins that also contain one or more copes of this novel domain, which we call the PKD domain. Whereas all known extracellular modules seem to be restricted to higher organisms, and the few exceptions seem to be evolutionary accidents, it was found that the PKD domain in extracellular parts of proteins from animals, eubacteria and archeabacteria. The animal proteins containing an individual PKD domain are heavily glycosylated, melanoma-associated cell surface proteins, such as melanocyte-specific human pmel17

(Kwon BS., 1993, J. Invest. Derm. (Supplement) 100:134-140), the MMP 115 protein (Mochii, M., Agata, K. and Eguchi, G., 1991, Pigment Cell Res. 4:41-47), and the nmb protein (Weterman, M.A.J., Ajubi, N., van Dinter, I. Degen, W., van Muijen, G., Ruiter D.J. and Bloemers, H.P.J., 1995, Int. J. Cancer 60:73-81). The physiological functions of these glycoproteins remains to be elucidated. Four enbacterial extracellular enzymers, three distinct collagenases and lysine-specific achromobacter protease I (API) also contain a single copy of the domain adjacent to their catalytic domains. Curiously, the highest degree of similarity between the collagenases is in the PKD domain. This may suggest that the domain in eukaryotic cells is involved in binding to collagenous domains. Four copies of the PKD domain are also present in the surface layer protein (SlpB) from *methanothermus*. The SlpB protein is (as is the PMEL17 family) heavily glycosylated and is predicted to be a glycoprotein component of the surface layer.

The PKD domain is predicted to be a globular domain that contains an antiparallel β-sheet. Although the PKD domains do not contain conserved cysteines, we believe they are extracellular domains because: 1) all identified homologues are extracellular or the PKD domain is in the extracellular part; 2) the first domain (amino add 281-353) is located between other known extracellular modules; and 3) there are no predicted transmembrane regions between the other identified (extracellular) modules and the 13 remaining FKD domains. Whereas the PKD domains in SlpB are very similar, pointing to rather recent duplication; the 14 domains in PKD1 are rather divergent. Even the most conserved (WDFGDG) motif (Fig. 7A-7B) is considerably modified in some of the PKD domains. Therefore, it is unlikely that unequal recombination between genomic sequences for motifs is a common source of mutations in this disease.

Although, it was not possible to identify specific domains in the C-terminal half of the protein, a long region was found which contained similarity to a putative *C. elegans*

Chromosome III protein (accession number Z48544). A hydrophobic stretch of 60 amino acids from 3986 to 4045 might represent a possible transmembrane domain, but without any clear resemblance to other such domains.

11.2.4. IDENTIFICATION OF AN ADPKD-CAUSING MUTATION

SSCP analysis was performed on samples obtained from 60 patients, as described, above, in Section 10.1. One variant ADPKD individual was identified via SSCP. Upon reamplification of amplified DNA from this individual (see Section 10.1, above), it was revealed that the patient contained a C to T transition at base pair 10,601 (exon 32) of the full-length PKD1 transcript. This mutation created a stop codon (TAG) at PKD1 amino acid position 765 which previously coded for a glutamine (CAG), thus truncating the final 728 amino acid residues which are normally present at the carboxy end of the PKD1 protein and yielding a final mutant protein of 3576 amino acids. The mutation was also predicted to create a novel Sty-1 site (CCCTAG); genomic DNA spanning this exon was amplified as before from the patient, his parents, and over 60 other unrelated individuals (120 alleles). After Sty-1 digestion, only the patient ZC (#118) was heterozygous for an enzyme site. The absence of the sequence change in over 120 alleses establishes this is not a polymorphic variation. The absence of the site in either parent establishes this as a new mutation, which corelates with the appearance of disease. Finally, the predicted impact on the protein (truncation) by itself is highly suggestive that it would impair or alter its function. This evidence, even in the absence of examination of the remainer of the gene or transcript in this patient, would be considered generally to be sufficient proof that this mutation is the cause of the disease.

12. DEPOSIT OF MICROORGANISMS

The following microorganisms were deposited with the American Type Culture Collection, Rockville, Maryland on May 27, 1994 and assigned the indicated accession numbers:

Microorganism	ATCC Accession No.
KG8	69636
cGGG10	69634
cDEB11	69635

The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 93, lines 1-20 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet

Name of depositary institution

American Type Culture Collection

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, MD 20852
US

Date of deposit May 27, 1994 Accession Number 69636

B. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on a separate attached sheet

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [illegible]

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the International application when filed (to be checked by the receiving Office)

02 JUN 1995

[illegible]
PCT [illegible] Division
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

International Application No: PCT/ /

Form PCT/RO/134 (cont.)

American Type Culture Collection

12301 Parklawn Drive
Rockville, MD 20852
US

Accession No.	Date of Deposit
69634	May 27, 1994
69635	May 27, 1994

WHAT IS CLAIMED IS:

1. An isolated nucleic acid containing a nucleotide sequence which encodes a polycystic kidney disease (PKD1) gene product.

2. The isolated nucleic acid of Claim 1 which encodes the amino acid sequence (SEQ ID NO: 2) of the PKD1 gene product depicted in FIG. 6.

3. The isolated nucleic acid of Claim 1 wherein the nucleotide sequence is the nucleotide sequence (SEQ ID NO: 1) depicted in FIg. 6.

4. The isolated nucleic acid Claim 1 which hybridizes under stringent conditions to the complement of the coding sequence of the nucleotide sequence depicted in FIG. 6 (SEQ ID NO: 1), or which hybridizes under less stringent conditions and encodes a functionally equivalent PKD1 gene product.

5. A nucleic acid vector containing the nucleotide sequence of Claim 1, 2, 3 or 4.

6. An expression vector containing the nucleotide sequence of Claim 1, 2, 3 or 4 in operative association with a nucleotide regulatory element that controls expression of the nucleotide sequence in a host cell.

7. An antisense molecule containing the nucleotide sequence of Claim 4.

8. A ribozyme molecule containing the nucleotide sequence of Claim 4.

9. A triple helix molecule containing the nucleotide sequence of Claim 4.

10. The nucleotide vector of Claim 5 which is a plasmid vector.

11. The nucleotide vector of Claim 5 which is a viral vector.

12. A genetically engineered host cell containing the nucleotide sequence of Claim 1, 2, 3 or 4.

13. A genetically engineered host cell containing the nucleotide sequence of Claim 1, 2, 3 or 4 in operative association with a regulatory element that controls expression of the nucleotide sequence in the host cell.

14. A substantially pure PKD1 gene product.

15. The substantially pure PKD1 gene product of Claim 14 wherein the gene product contains the amino acid sequence (SEQ ID NO: 2) depicted in FIG. 6.

15. An antibody that immunospecifically binds to a PKD1 gene product.

16. A method for diagnosing autosomal dominant polycystic kidney disease, comprising detecting a mutant PKD1 gene or gene product in a patient sample.

17. A method for treating autosomal dominant polycystic kidney disease, comprising administering an effective amount of a compound to a patient in need of such treatment, which compound inhibits the synthesis, expression or activity of a mutant PKD1 gene product.

18. The method of Claim 17 in which the compound is an antisense or ribozyme molecule that blocks translation of mutant PKD1 mRNA.

19. The method of Claim 18 in which the compound is a nucleotide that is complementary to the 5' region of the PKD1 gene, and blocks transcription of the PKD1 gene via triple helix formation.

20. The method of Claim 19 further comprising replacing the mutant PKD1 gene with a normal allele, or replacing the mutant PKD1 gene product with a normal PKD1 gene product.

21. The method of Claim 19 in which the compound is an antibody that immunospecifically binds and inactivates the mutant PKD1 gene product.

22. A method for treating autosomal dominant polycystic kidney disease, comprising administering a normal allele of the PKD1 gene to a patient in need of such treatment, so that the normal PKD1 allele is expressed in the patient.

23. A method for treating autosomal dominant polycystic kidney disease, comprising administering an effective amount of a normal PKD1 gene product to a patient in need of such therapy.

24. A method of measuring the presence of a PKD1 gene product in a sample, comprising:

(a) contacting the sample suspected of containing a PKD1 gene product with an antibody that binds to the PKD1 gene product under conditions which allow for the formation of reaction complexes comprising the antibody and the PKD1 gene product;

(b) detecting the formation of reaction complexes comprising the antibody and PKD1 gene product in the sample, in which detection of the formation of reaction complexes indicates the presence of the PKD1 gene product in the sample.

25. The method of Claim 24 in which the antibody is bound to a solid phase support.

26. The method of Claim 24 in which the PKD1 gene product is bound to a solid phase support.

27. The method of Claim 25 or 26 which additionally comprises contacting the sample with a labeled PKD1 gene product in step (a), and removing unbound substances prior to step (b), in which a decrease in the amount of reaction complexes comprising the antibody and the labelled PKD1 gene product indicates the presence of the PKD1 gene product in the sample.

28. A method of evaluating the level of PKD1 gene product in a biological sample comprising:

(a) detecting the formation of reaction complexes in a biological sample according to the method of Claim 24; and

(b) evaluating the amount of reaction complexes formed, which amount of reaction complexes corresponds to the level of PKD1 gene product in the biological sample.

29. A method of detecting or diagnosing the presence of a disease associated with elevated or decreased levels of PKD1 gene product in a mammalian subject comprising:

(a) evaluating the level of PKD1 gene product in a biological sample from mammalian subject according to Claim 28; and

(b) comparing the level detected in step (a) to a level of PKD1 gene product present in normal subjects or in the subject at an earlier time, in which an increase or a decrease in the level of the PKD1 gene product as compared to normal levels indicates a disease condition.

30. A method for monitoring a therapeutic treatment of a disease associated with elevated or decreased levels of PKD1 gene product in a mammalian subject, comprising evaluating the levels of the PKD1 gene product in a series of biological samples obtained at different time points from a mammalian subject undergoing a therapeutic treatment for a disease associated with elevated or decreased levels of PKD1 gene product, according to the method of Claim 28.

31. The method according to Claim 29 or 30 wherein the disease associated with decreased levels of PKD1 gene product is selected from the group consisting of polycystic kidney disease, and acquired cystic disease.

32. A test kit for measuring the presence of or amount of PKD1 gene product in a sample, comprising

(a) an antibody that immunospecifically binds to a PKD1 gene product;
(b) means for detecting binding of the anti-PKD1 gene product antibody to PKD1 gene product in a sample;
(c) other reagents; and
(d) directions for use of the kit.

33. A pharmaceutical composition for treating polycystic kidney disease in a mammal, comprising the PKD1 gene product of Claim 14 and a pharmaceutically acceptable carrier.

34. A method for treating polycystic kidney disease in a mammal comprising administering an amount of a pharmaceutical composition of Claim 33 effective to ameliorate the symptoms of polycystic kidney disease.

35. A method for treating polycystic kidney disease in a mammal comprising increasing the expression of a protein encoded by the nucleic acid of Claim 1, 2, 3 or 4.

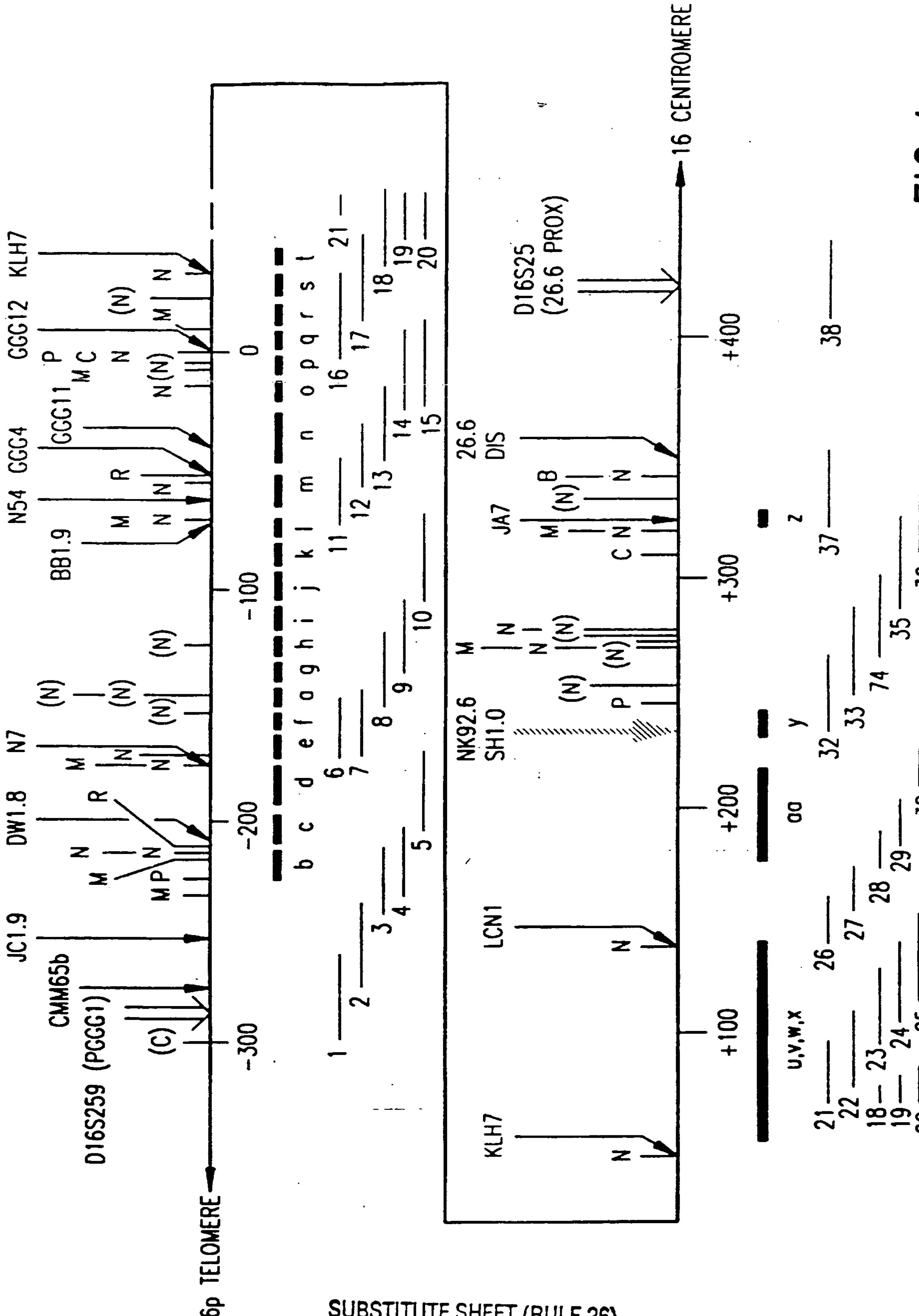

16p TELOMERE
16 CENTROMERE
D16S259 (PGGG1)
CMM65b
JC1.9
DW1.8
N7
BB1.9
N54
GGG4
GGG11
GGG12
KLH7
D16S25
(26.6 PROX)
26.6
DIS
JA7
NK92.6
SH1.0
LCN1
KLH7
-300
-200
-100
0
+100
+200
+300
+400
u,v,w,x
aa
y
z

FIG.1

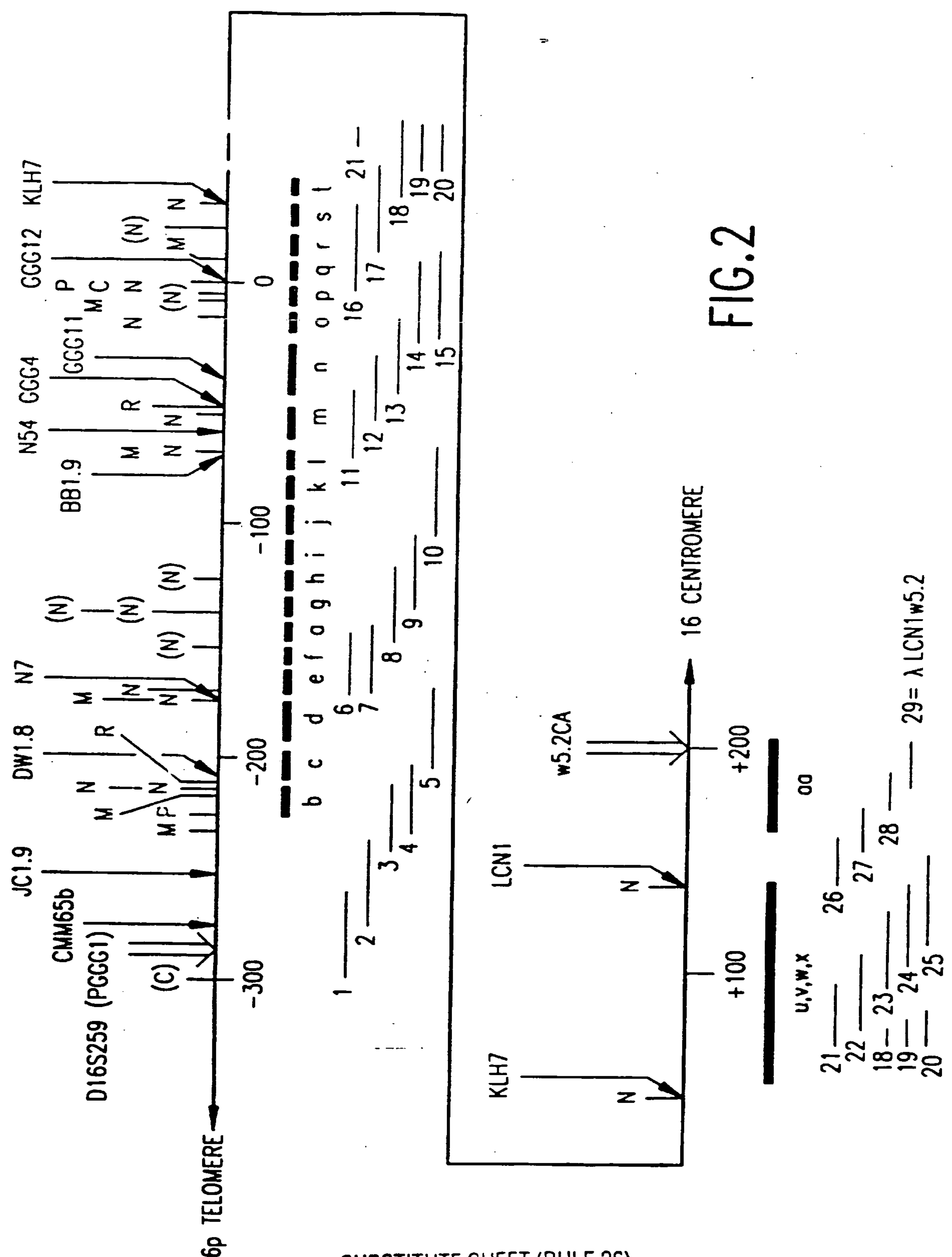

16p TELOMERE
D16S259 (PGGG1)
CMM65b
JC1.9
DW1.8
N7
BB1.9
N54
GGG4
GGG11
GGG12
KLH7
-300
-200
-100
0
LCN1
w5.2CA
16 CENTROMERE
+100
+200
u,v,w,x
aa
29= λLCN1w5.2

FIG.2

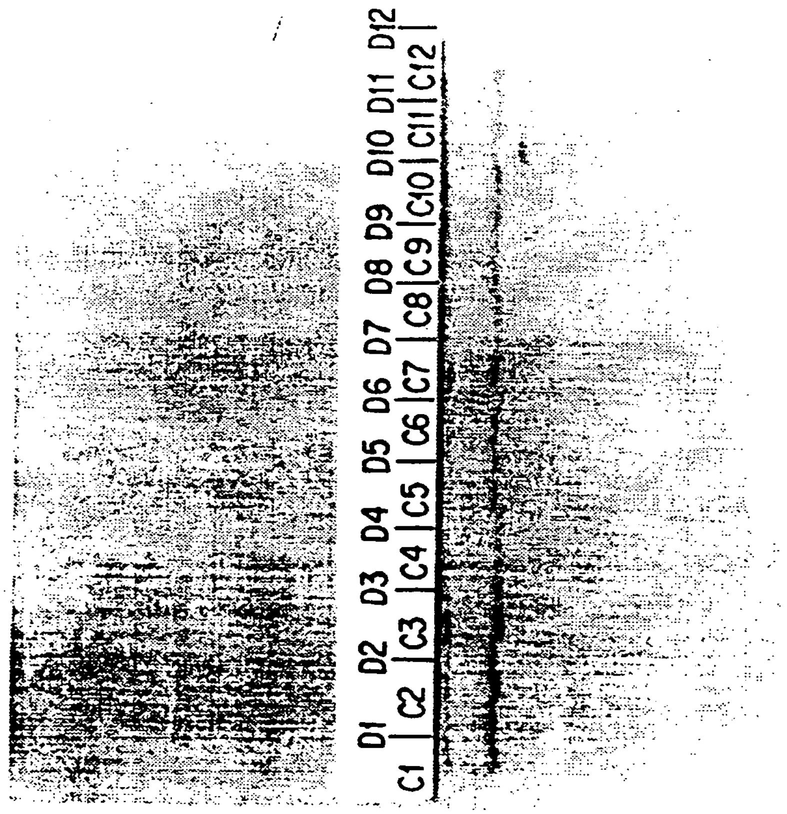

D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12
C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12

FIG.3A

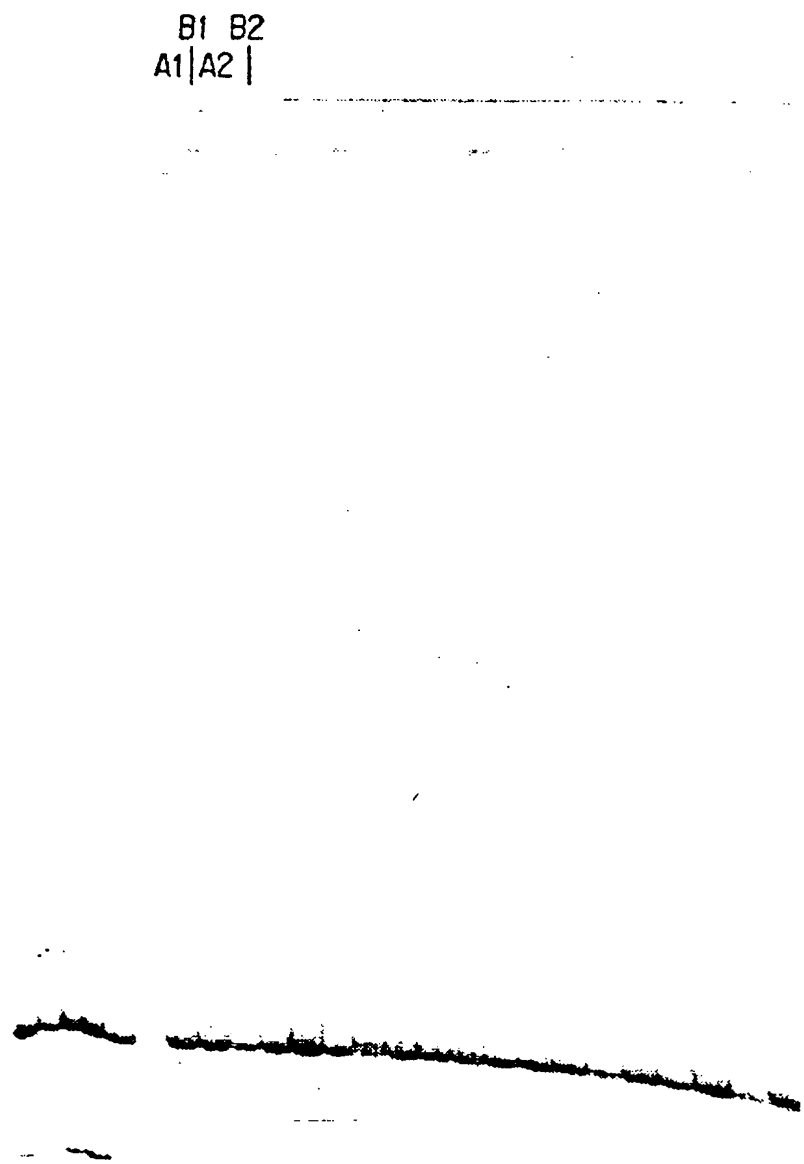
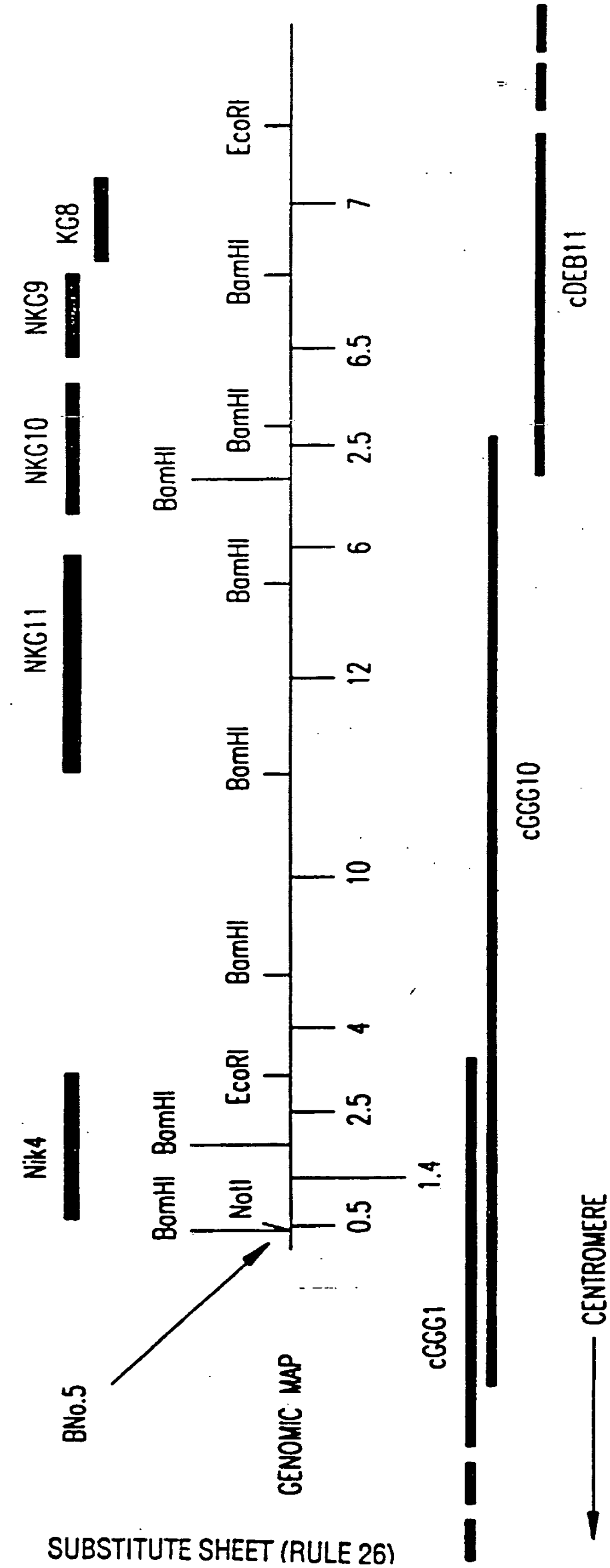


FIG.3B



BNo.5
GENOMIC MAP
BamHI
NotI
BamHI
BamHI
EcoRI
BamHI
BamHI
BamHI
BamHI
BamHI
BamHI
EcoRI
0.5
1.4
2.5
4
10
12
6
2.5
6.5
7
Nik4
NKG11
NKG10
NKG9
KG8
cGGG1
cGGG10
cDEB11
CENTROMERE

FIG.4

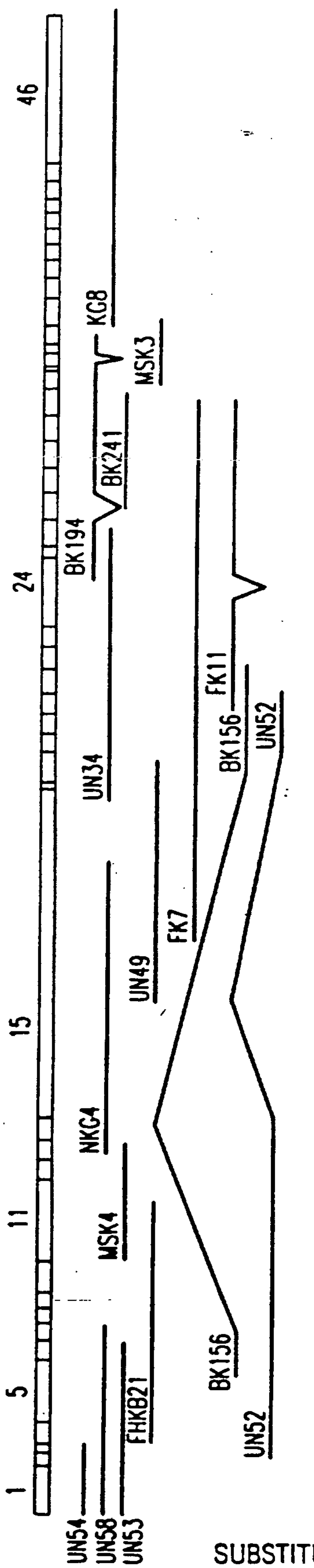

1
5
11
15
24
46
UN54
UN58
UN53
FHKB21
MSK4
NKG4
UN49
FK7
BK156
UN52
BK156
UN52
FK11
UN34
BK194
BK241
MSK3
KG8

FIG.5A

	SIZE	FUN53	FUN54	FUN59	FUN52	FHKB21	NKG11	FUN49	FUN34	BK156	FK7	FK11	BK194	BK241	KG8	cDNAPCR
EXON1	443	X	X	X												
EXON2	72	X	X	X												
EXON3	72	X	X	X												
EXON4	170	X	X	X	X											
EXON5	672	X	X	X	X	X				X						
EXON6	184	X	X	X	X	X				X						
EXON7	221				X	X										
EXON7A	163									X						
EXON8	116				X	X										
EXON9	127				X	X										
EXON10	248				X	X										
EXON11	756				X	X										
EXON12	136				X											X
EXON12A	132															
EXON13	176				X		X									X
EXON14	134				X		X									X
EXON15	3617						X	X			X					
EXON15A	89				X											
EXON15B	327									X						
EXON16	153							X	X	X	X					
EXON17	172								X	X						
EXON18	144				X				X	X	X					
EXON19	280				X				X	X	X					
EXON20	214				X				X	X	X	X				
EXON21	160								X		X	X				
EXON22	153								X		X	X				
EXON23	145								X		X	X				
EXON24	630								X		X		X			

(CONT. ON FIG. 5C)

FIG.5B

(FROM FIG. 5B)

	SIZE	FUN53	FUN54	FUN59	FUN52	FHKB21	NKG11	FUN49	FUN34	BK156	FK7	FK11	BK194	BK241	KG8	cDNAPCR
EXON24A	380											X				
EXON24B	125											X				
EXON25	157								X		X	X	X			
EXON26	253										X	X	X			
EXON27	196										X	X		X		
EXON28	171												X	X		
EXON29	144												X	X		
EXON30	211												X	X		
EXON31	127												X	X		
EXON32	117												X	X		X
EXON33	53												X			X
EXON33A	50															X
EXON34	185															X
EXON35	94												X			X
EXON36	119														X	X
EXON37	203														X	
EXON38	195														X	
EXON39	140														X	
EXON40	113														X	
EXON41	157														X	
EXON42	111														X	
EXON43	175														X	
EXON44	292														X	
EXON45	135														X	
EXON46	1770															
BOLD	DENOTES EXONS NOT INCLUDED IN FINAL PROTEIN															

FIG.5C

1 ATG CCG CCC GCC GCG CCC GCC CGC CTG GCG CTG GCC CTG GGC CTG GGC CTG TGG CTC GGG 60
1 M P P A A P A R L A L A L G L G L W L G 20

61 GCG CTG GCG GGG GGG CCC GGG CGC GGC TGC GGG CCC TGC GAG CCC CCC TGC CTC TGC GGG 120
21 A L A G G P G R G C G P C E P P C L C G 40

121 CCA GCG CCC GGC GCC GCC TGC CGC GTC AAC TGC TCG GGC CGC GGG CTG CGG ACG CTC GGT 180
41 P A P G A A C R V N C S G R G L R T L G 60

181 CCC GCG CTG CGC ATC CCC GCG GAC GCC ACA GAG CTA GAC GTC TCC CAC AAC CTG CTC CGG 240
61 P A L R I P A D A T E L D V S H N L L R 80

241 GCG CTG GAC GTT GGG CTC CTG GCG AAC CTC TCG GCG CTG GCA GAG CTG GAT ATA AGC AAC 300
81 A L D V G L L A N L S A L A E L D I S N 100

301 AAC AAG ATT TCT ACG TTA GAA GAA GGA ATA TTT GCT AAT TTA TTT AAT TTA AGT GAA ATA 360
101 N K I S T L E E G I F A N L F N L S E I 120

361 AAC CTG AGT GGG AAC CCG TTT GAG TGT GAC TGT GGC CTG GCG TGG CTG CCG CAA TGG GCG 420
121 N L S G N P F E C D C G L A W L P Q W A 140

421 GAG GAG CAG CAG GTG CGG GTG GTG CAG CCC GAG GCA GCC ACG TGT GCT GGG CCT GGC TCC 480
141 E E Q Q V R V V Q P E A A T C A G P G S 160

481 CTG GCT GGC CAG CCT CTG CTT GGC ATC CCC TTG CTG GAC AGT GGC TGT GGT GAG GAG TAT 540
161 L A G Q P L L G I P L L D S G C G E E Y 180

541 GTC GCC TGC CTC CCT GAC AAC AGC TCA GGC ACC GTG GCA GCA GTG TCC TTT TCA GCT GCC 600
181 V A C L P D N S S G T V A A V S F S A A 200

601 CAC GAA GGC CTG CTT CAG CCA GAG GCC TGC AGC GCC TTC TGC TTC TCC ACC GGC CAG GGC 660
201 H E G L L Q P E A C S A F C F S T G Q G 220

661 CTC GCA GCC CTC TCG GAG CAG GGC TGG TGC CTG TGT GGG GCG GCC CAG CCC TCC AGT GCC 720
221 L A A L S E Q G W C L C G A A Q P S S A 240

721 TCC TTT GCC TGC CTG TCC CTC TGC TCC GGG CCC CCG GCA CCT CCT GCC CCC ACC TGT AGG 780
241 S F A C L S L C S G P P A P P A P T C R 260

781 GGC CCC ACC CTC CTC CAG CAC GTC TTC CCT GCC TCC CCA GGG GCC ACC CTG GTG GGG CCC 840
261 G P T L L Q H V F P A S P G A T L V G P 280

FIG.6A

841 CAC GGA CCT CTG GCC TCT GGC CAG CTA GCA GCC TTC CAC ATC GCT GCC CCG CTC CCT GTC 900
281 H G P L A S G Q L A A F H I A A P L P V 300

901 ACT GAC ACA CGC TGG GAC TTC GGA GAC GGC TCC GCC GAG GTG GAT GCC GCT GGG CCG GCT 960
301 T D T R W D F G D G S A E V D A A G P A 320

961 GCC TCG CAT CGC TAT GTG CTG CCT GGG CGC TAT CAC GTG ACG GCC GTG CTG GCC CTG GGG 1020
321 A S H R Y V L P G R Y H V T A V L A L G 340

1021 GCC GGC TCA GCC CTG CTG GGG ACA GAC GTG CAG GTG GAA GCG GCA CCT GCC GCC CTG GAG 1080
341 A G S A L L G T D V Q V E A A P A A L E 360

1081 CTC GTG TGC CCG TCC TCG GTG CAG AGT GAC GAG AGC CTC GAC CTC AGC ATC CAG AAC CGC 1140
361 L V C P S S V Q S D E S L D L S I Q N R 380

1141 GGT GGT TCA GGC CTG GAG GCC GCC TAC AGC ATC GTG GCC CTG GGC GAG GAG CCG GCC CGA 1200
381 G G S G L E A A Y S I V A L G E E P A R 400

1201 GCG GTG CAC CCG CTC TGC CCC TCG GAC ACG GAG ATC TTC CCT GGC AAC GGG CAC TGC TAC 1260
401 A V H P L C P S D T E I F P G N G H C Y 420

1261 CGC CTG GTG GTG GAG AAG GCG GCC TGG CTG CAG GCG CAG GAG CAG TGT CAG GCC TGG GCC 1320
421 R L V V E K A A W L Q A Q E Q C Q A W A 440

1321 GGG GCC GCC CTG GCA ATG GTG GAC AGT CCC GCC GTG CAG CGC TTC CTG GTC TCC CGG GTC 1380
441 G A A L A M V D S P A V Q R F L V S R V 460

1381 ACC AGG AGC CTA GAC GTG TGG ATC GGC TTC TCG ACT GTG CAG GGG GTG GAG GTG GGC CCA 1440
461 T R S L D V W I G F S T V Q G V E V G P 480

1441 GCG CCG CAG GGC GAG GCC TTC AGC CTG GAG AGC TGC CAG AAC TGG CTG CCC GGG GAG CCA 1500
481 A P Q G E A F S L E S C Q N W L P G E P 500

1501 CAC CCA GCC ACA GCC GAG CAC TGC GTC CGG CTC GGG CCC ACC GGG TGG TGT AAC ACC GAC 1560
501 H P A T A E H C V R L G P T G W C N T D 520

1561 CTG TGC TCA GCG CCG CAC AGC TAC GTC TGC GAG CTG CAG CCC GGA GGC CCA GTG CAG GAT 1620
521 L C S A P H S Y V C E L Q P G G P V Q D 540

1621 GCC GAG AAC CTC CTC GTG GGA GCG CCC AGT GGG GAC CTG CAG GGA CCC CTG ACG CCT CTG 1680
541 A E N L L V G A P S G D L Q G P L T P L

FIG.6B

1681 GCA CAG CAG GAC GGC CTC TCA GCC CCG CAC GAG CCC GTG GAG GTC ATG GTA TTC CCG GGC 1740
561 A Q Q D G L S A P H E P V E V M V F P G 580

1741 CTG CGT CTG AGC CGT GAA GCC TTC CTC ACC ACG GCC GAA TTT GGG ACC CAG GAG CTC CGG 1800
581 L R L S R E A F L T T A E F G T Q E L R 600

1801 CGG CCC GCC CAG CTG CGG CTG CAG GTG TAC CGG CTC CTC AGC ACA GCA GGG ACC CCG GAG 1860
601 R P A Q L R L Q V Y R L L S T A G T P E 620

1861 AAC GGC AGC GAG CCT GAG AGC AGG TCC CCG GAC AAC AGG ACC CAG CTG GCC CCC GCG TGC 1920
621 N G S E P E S R S P D N R T Q L A P A C 640

1921 ATG CCA GGG GGA CGC TGG TGC CCT GGA GCC AAC ATC TGC TTG CCG CTG GAC GCC TCC TGC 1980
641 M P G G R W C P G A N I C L P L D A S C 660

1981 CAC CCC CAG GCC TGC GCC AAT GGC TGC ACG TCA GGG CCA GGG CTA CCC GGG GCC CCC TAT 2040
661 H P Q A C A N G C T S G P G L P G A P Y 680

2041 GCG CTA TGG AGA GAG TTC CTC TTC TCC GTT CCC GCG GGG CCC CCC GCG CAG TAC TCG GTC 2100
681 A L W R E F L F S V P A G P P A Q Y S V 700

2101 ACC CTC CAC GGC CAG GAT GTC CTC ATG CTC CCT GGT GAC CTC GTT GGC TTG CAG CAC GAC 2160
701 T L H G Q D V L M L P G D L V G L Q H D 720

2161 GCT GGC CCT GGC GCC CTC CTG CAC TGC TCG CCG GCT CCC GGC CAC CCT GGT CCC CGG GCC 2220
721 A G P G A L L H C S P A P G H P G P R A 740

2221 CCG TAC CTC TCC GCC AAC GCC TCG TCA TGG CTG CCC CAC TTG CCA GCC CAG CTG GAG GGC 2280
741 P Y L S A N A S S W L P H L P A Q L E G 760

2281 ACT TGG CGC TGC CCT GCC TGT GCC CTG CGG CTG CTT GCA CAA CGG GAA CAG CTC ACC GTG 2340
761 T W G C P A C A L R L L A Q R E Q L T V 780

2341 CTG CTG GGC TTG AGG CCC AAC CCT GGA CTG CGG CTG CCT GGG CGC TAT GAG GTC CGG GCA 2400
781 L L G L R P N P G L R L P G R Y E V R A 800

2401 GAG GTG GGC AAT GGC GTG TCC AGG CAC AAC CTC TCC TGC AGC TTT GAC GTG GTC TCC CCA 2460
801 E V G N G V S R H N L S C S F D V V S P 820

2461 GTG GCT GGG CTG CGG GTC ATC TAC CCT GCC CCC CGC GAC GGC CGC CTC TAC GTG CCC ACC 2520
821 V A G L R V I Y P A P R D G R L Y V P T 840

FIG.6C

2521 AAC GGC TCA GCC TTG GTG CTC CAG GTG GAC TCT GGT GCC AAC GCC ACG GCC ACG GCT CGC 2580
841 N G S A L V L Q V D S G A N A T A T A R 860

2581 TGG CCT GGG GGC AGT CTC AGC GCC CGC TTT GAG AAT GTC TGC CCT GCC CTG GTG GCC ACC 2640
861 W P G G S L S A R F E N V C P A L V A T 880

2641 TTC GTG CCC GCC TGC CCC TGG GAG ACC AAC GAT ACC CTG TTC TCA GTG GTA GCA CTG CCG 2700
881 F V P A C P W E T N D T L F S V V A L P 900

2701 TGG CTC AGT GAG GGG GAG CAC GTG GTG GAC GTG GTG GTG GAA AAC AGC GCC AGC CGG GCC 2760
901 W L S E G E H V V D V V V E N S A S R A 920

2761 AAC CTC AGC CTG CGG GTG ACG GCG GAG GAG CCC ATC TGT GGC CTC CGC GCC ACG CCC AGC 2820
921 N L S L R V T A E E P I C G L R A T P S 940

2821 CCC GAG GCC CGT GTA CTG CAG GGA GTC CTA GTG AGG TAC AGC CCC GTG GTG GAG GCC GGC 2880
941 P E A R V L Q G V L V R Y S P V V E A G 960

2881 TCG GAC ATG GTC TTC CGG TGG ACC ATC AAC GAC AAG CAG TCC CTG ACC TTC CAG AAC GTG 2940
961 S D M V F R W T I N D K Q S L T F Q N V 980

2941 GTC TTC AAT GTC ATT TAT CAG AGC GCG GCG GTC TTC AAG CTC TCA CTG ACG GCC TCC AAC 3000
981 V F N V I Y Q S A A V F K L S L T A S N 1000

3001 CAC GTG AGC AAC GTC ACC GTG AAC TAC AAC GTA ACC GTG GAG CGG ATG AAC AGG ATG CAG 3060
1001 H V S N V T V N Y N V T V E R M N R M Q 1020

3061 GGT CTG CAG GTC TCC ACA GTG CCG GCC GTG CTG TCC CCC AAT GCC ACG CTA GCA CTG ACG 3120
1021 G L Q V S T V P A V L S P N A T L A L T 1040

3121 GCG GGC GTG CTG GTG GAC TCG GCC GTG GAG GTG GCC TTC CTG TGG ACC TTT GGG GAT GGG 3180
1041 A G V L V D S A V E V A F L W T F G D G 1060

3181 GAG CAG GCC CTC CAC CAG TTC CAG CCT CCG TAC AAC GAG TCC TTC CCA GTT CCA GAC CCC 3240
1061 E Q A L H Q F Q P P Y N E S F P V P D P 1080

3241 TCG GTG GCC CAG GTG CTG GTG GAG CAC AAT GTC ACG CAC ACC TAC GCT GCC CCA GGT GAG 3300
1081 S V A Q V L V E H N V T H T Y A A P G E 1100

FIG.6D

3301 TAC CTC CTG ACC GTG CTG GCA TCT AAT GCC TTC GAG AAC CTG ACC CAG CAG GTG CCT GTG 3360
1101 Y L L T V L A S N A F E N L T Q Q V P V 1120

3361 AGC GTG CGC GCC TCC CTG CCC TCC GTG GCT GTG GGT GTG AGT GAC GGC GTC CTG GTG GCC 3420
1121 S V R A S L P S V A V G V S D G V L V A 1140

3421 GGC CGG CCC GTC ACC TTC TAC CCG CAC CCG CTG CCC TCG CCT GGG GGT GTT CTT TAC ACG 3480
1141 G R P V T F Y P H P L P S P G G V L Y T 1160

3481 TGG GAC TTC GGG GAC GGC TCC CCT GTC CTG ACC CAG AGC CAG CCG GCT GCC AAC CAC ACC 3540
1161 W D F G D G S P V L T Q S Q P A A N H T 1180

3541 TAT GCC TCG AGG GGC ACC TAC CAC GTG CGC CTG GAG GTC AAC AAC ACG GTG AGC GGT GCG 3600
1181 Y A S R G T Y H V R L E V N N T V S G A 1200

3601 GCG GCC CAG GCG GAT GTG CGC GTC TTT GAG GAG CTC CGC GGA CTC AGC GTG GAC ATG AGC 3660
1201 A A Q A D V R V F E E L R G L S V D M S 1220

3661 CTG GCC GTG GAG CAG GGC GCC CCC GTG GTG GTC AGC GCC GCG GTG CAG ACG GGC GAC AAC 3720
1221 L A V E Q G A P V V V S A A V Q T G D N 1240

3721 ATC ACG TGG ACC TTC GAC ATG GGG GAC GGC ACC GTG CTG TCG GGC CCG GAG GCA ACA GTG 3780
1241 I T W T F D M G D G T V L S G P E A T V 1260

3781 GAG CAT GTG TAC CTG CGG GCA CAG AAC TGC ACA GTG ACC GTG GGT GCG GGC AGC CCC GCC 3840
1261 E H V Y L R A Q N C T V T V G A G S P A 1280

3841 GGC CAC CTG GCC CGG AGC CTG CAC GTG CTG GTC TTC GTC CTG GAG GTG CTG CGC GTT GAA 3900
1281 G H L A R S L H V L V F V L E V L R V E 1300

3901 CCC GCC GCC TGC ATC CCC ACG CAG CCT GAC GCG CGG CTC ACG GCC TAC GTC ACC GGG AAC 3960
1301 P A A C I P T Q P D A R L T A Y V T G N 1320

3961 CCG GCC CAC TAC CTC TTC GAC TGG ACC TTC GGG GAT GGC TCC TCC AAC ACG ACC GTG CGG 4020
1321 P A H Y L F D W T F G D G S S N T T V R 1340

4021 GGG TGC CCG ACG GTG ACA CAC AAC TTC ACG CGG AGC GGC ACG TTC CCC CTG GCG CTG GTG 4080
1341 G C P T V T H N F T R S G T F P L A L V 1360

FIG.6E

4081 CTG TCC AGC CGC GTG AAC AGG GCG CAT TAC TTC ACC AGC ATC TGC GTG GAG CCA GAG GTG 4140
1361 L S S R V N R A H Y F T S I C V E P E V 1380

4141 GGC AAC GTC ACC CTG CAG CCA GAG AGG CAG TTT GTG CAG CTC GGG GAC GAG GCC TGG CTG 4200
1381 G N V T L Q P E R Q F V Q L G D E A W L 1400

4201 GTG GCA TGT GCC TGG CCC CCG TTC CCC TAC CGC TAC ACC TGG GAC TTT GGC ACC GAG GAA 4260
1401 V A C A W P P F P Y R Y T W D F G T E E 1420

4261 GCC GCC CCC ACC CGT GCC AGG GGC CCT GAG GTG ACG TTC ATC TAC CGA GAC CCA GGC TCC 4320
1421 A A P T R A R G P E V T F I Y R D P G S 1440

4321 TAT CTT GTG ACA GTC ACC GCG TCC AAC AAC ATC TCT GCT GCC AAT GAC TCA GCC CTG GTG 4380
1441 Y L V T V T A S N N I S A A N D S A L V 1460

4381 GAG GTG CAG GAG CCC GTG CTG GTC ACC AGC ATC AAG GTC AAT GGC TCC CTT GGG CTG GAG 4440
1461 E V Q E P V L V T S I K V N G S L G L E 1480

4441 CTG CAG CAG CCG TAC CTG TTC TCT GCT GTG GGC CGT GGG CGC CCC GCC AGC TAC CTG TGG 4500
1481 L Q Q P Y L F S A V G R G R P A S Y L W 1500

4501 GAT CTG GGG GAC GGT GGG TGG CTC GAG GGT CCG GAG GTC ACC CAC GCT TAC AAC AGC ACA 4560
1501 D L G D G G W L E G P E V T H A Y N S T 1520

4561 GGT GAC TTC ACC GTT AGG GTG GCC GGC TGG AAT GAG GTG AGC CGC AGC GAG GCC TGG CTC 4620
1521 G D F T V R V A G W N E V S R S E A W L 1540

4621 AAT GTG ACG GTG AAG CGG CGC GTG CGG GGG CTC GTC GTC AAT GCA AGC CGC ACG GTG GTG 4680
1541 N V T V K R R V R G L V V N A S R T V V 1560

4681 CCC CTG AAT GGG AGC GTG AGC TTC AGC ACG TCG CTG GAG GCC GGC AGT GAT GTG CGC TAT 4740
1561 P L N G S V S F S T S L E A G S D V R Y 1580

4741 TCC TGG GTG CTC TGT GAC CGC TGC ACG CCC ATC CCT GGG GGT CCT ACC ATC TCT TAC ACC 4800
1581 S W V L C D R C T P I P G G P T I S Y T 1600

4801 TTC CGC TCC GTG GGC ACC TTC AAT ATC ATC GTC ACG GCT GAG AAC GAG GTG GGC TCC GCC 4860
1601 F R S V G T F N I I V T A E N E V G S A 1620

FIG.6F

4861 CAG GAC AGC ATC TTC GTC TAT GTC CTG CAG CTC ATA GAG GGG CTG CAG GTG GTG GGC GGT 4920
1621 Q D S I F V Y V L Q L I E G L Q V V G G 1640

4921 GGC CGC TAC TTC CCC ACC AAC CAC ACG GTA CAG CTG CAG GCC GTG GTT AGG GAT GGC ACC 4980
1641 G R Y F P T N H T V Q L Q A V V R D G T 1660

4981 AAC GTC TCC TAC AGC TGG ACT GCC TGG AGG GAC AGG GGC CCG GCC CTG GCC GGC AGC GGC 5040
1661 N V S Y S W T A W R D R G P A L A G S G 1680

5041 AAA GGC TTC TCG CTC ACC GTG CTC GAG GCC GGC ACC TAC CAT GTG CAG CTG CGG GCC ACC 5100
1681 K G F S L T V L E A G T Y H V Q L R A T 1700

5101 AAC ATG CTG GGC AGC GCC TGG GCC GAC TGC ACC ATG GAC TTC GTG GAG CCT GTG GGG TGG 5160
1701 N M L G S A W A D C T M D F V E P V G W 1720

5161 CTG ATG GTG GCC GCC TCC CCG AAC CCA GCT GCC GTC AAC ACA AGC GTC ACC CTC AGT GCC 5220
1721 L M V A A S P N P A A V N T S V T L S A 1740

5221 GAG CTG GCT GGT GGC AGT GGT GTC GTA TAC ACT TGG TCC TTG GAG GAG GGG CTG AGC TGG 5280
1741 E L A G G S G V V Y T W S L E E G L S W 1760

5281 GAG ACC TCC GAG CCA TTT ACC ACC CAT AGC TTC CCC ACA CCC GGC CTG CAC TTG GTC ACC 5340
1761 E T S E P F T T H S F P T P G L H L V T 1780

5341 ATG ACG GCA GGG AAC CCG CTG GGC TCA GCC AAC GCC ACC GTG GAA GTG GAT GTG CAG GTG 5400
1781 M T A G N P L G S A N A T V E V D V Q V 1800

5401 CCT GTG AGT GGC CTC AGC ATC AGG GCC AGC GAG CCC GGA GGC AGC TTC GTG GCG GCC GGG 5460
1801 P V S G L S I R A S E P G G S F V A A G 1820

5461 TCC TCT GTG CCC TTT TGG GGG CAG CTG GCC ACG GGC ACC AAT GTG AGC TGG TGC TGG GCT 5520
1821 S S V P F W G Q L A T G T N V S W C W A 1840

5521 GTG CCC GGC GGC AGC AGC AAG CGT GGC CCT CAT GTC ACC ATG GTC TTC CCG GAT GCT GGC 5580
1841 V P G G S S K R G P H V T M V F P D A G 1860

5581 ACC TTC TCC ATC CGG CTC AAT GCC TCC AAC GCA GTC AGC TGG GTC TCA GCC ACG TAC AAC 5640
1861 T F S I R L N A S N A V S W V S A T Y N 1880

FIG.6G

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5641 CTC ACG GCG GAG GAG CCC ATC GTG GGC CTG GTG CTG TGG GCC AGC AGC AAG GTG GTG GCG 5700
1881 L T A E E P I V G L V L W A S S K V V A 1900

5701 CCC GGG CAG CTG GTC CAT TTT CAG ATC CTG CTG GCT GCC GGC TCA GCT GTC ACC TTC CGC 5760
1901 P G Q L V H F Q I L L A A G S A V T F R 1920

5761 CTA CAG GTC GGC GGG GCC AAC CCC GAG GTG CTC CCC GGG CCC CGT TTC TCC CAC AGC TTC 5820
1921 L Q V G G A N P E V L P G P R F S H S F 1940

5821 CCC CGC GTC GGA GAC CAC GTG GTG AGC GTG CGG GGC AAA AAC CAC GTG AGC TGG GCC CAG 5880
1941 P R V G D H V V S V R G K N H V S W A Q 1960

5881 GCG CAG GTG CGC ATC GTG GTG CTG GAG GCC GTG AGT GGG CTG CAG GTG CCC AAC TGC TGC 5940
1961 A Q V R I V V L E A V S G L Q V P N C C 1980

5941 GAG CCT GGC ATC GCC ACG GGC ACT GAG AGG AAC TTC ACA GCC CGC GTG CAG CGC GGC TCT 6000
1981 E P G I A T G T E R N F T A R V Q R G S 2000

6001 CGG GTC GCC TAC GCC TGG TAC TTC TCG CTG CAG AAG GTC CAG GGC GAC TCG CTG GTC ATC 6060
2001 R V A Y A W Y F S L Q K V Q G D S L V I 2020

6061 CTG TCG GGC CGC GAC GTC ACC TAC ACG CCC GTG GCC GCG GGG CTG TTG GAG ATC CAG GTG 6120
2021 L S G R D V T Y T P V A A G L L E I Q V 2040

6121 CGC GCC TTC AAC GCC CTG GGC AGT GAG AAC CGC ACG CTG GTG CTG GAG GTT CAG GAC GCC 6180
2041 R A F N A L G S E N R T L V L E V Q D A 2060

6181 GTC CAG TAT GTG GCC CTG CAG AGC GGC CCC TGC TTC ACC AAC CGC TCG GCG CAG TTT GAG 6240
2061 V Q Y V A L Q S G P C F T N R S A Q F E 2080

6241 GCC GCC ACC AGC CCC AGC CCC CGG CGT GTG GCC TAC CAC TGG GAC TTT GGG GAT GGG TCG 6300
2081 A A T S P S P R R V A Y H W D F G D G S 2100

6301 CCA GGG CAG GAC ACA GAT GAG CCC AGG GCC GAG CAC TCC TAC CTG AGG CCT GGG GAC TAC 6360
2101 P G Q D T D E P R A E H S Y L R P G D Y 2120

6361 CGC GTG CAG GTG AAC GCC TCC AAC CTG GTG AGC TTC TTC GTG GCG CAG GCC ACG GTG ACC 6420
2121 R V Q V N A S N L V S F F V A Q A T V T 2140

FIG.6H

6421 GTC CAG GTG CTG GCC TGC CGG GAG CCG GAG GTG GAC GTG GTC CTG CCC CTG CAG GTG CTG 6480
2141 V Q V L A C R E P E V D V V L P L Q V L 2160

6481 ATG CGG CGA TCA CAG CGC AAC TAC TTG GAG GCC CAC GTT GAC CTG CGC GAC TGC GTC ACC 6540
2161 M R R S Q R N Y L E A H V D L R D C V T 2180

6541 TAC CAG ACT GAG TAC CGC TGG GAG GTG TAT CGC ACC GCC AGC TGC CAG CGG CCG GGG CGC 6600
2181 Y Q T E Y R W E V Y R T A S C Q R P G R 2200

6601 CCA GCG CGT GTG GCC CTG CCC GGC GTG GAC GTG AGC CGG CCT CGG CTG GTG CTG CCG CGG 6660
2201 P A R V A L P G V D V S R P R L V L P R 2220

6661 CTG GCG CTG CCT GTG GGG CAC TAC TGC TTT GTG TTT GTC GTG TCA TTT GGG GAC ACG CCA 6720
2221 L A L P V G H Y C F V F V V S F G D T P 2240

6721 CTG ACA CAG AGC ATC CAG GCC AAT GTG ACG GTG GCC CCC GAG CGC CTG GTG CCC ATC ATT 6780
2241 L T Q S I Q A N V T V A P E R L V P I I 2260

6781 GAG GGT GGC TCA TAC CGC GTG TGG TCA GAC ACA CGG GAC CTG GTG CTG GAT GGG AGC GAG 6840
2261 E G G S Y R V W S D T R D L V L D G S E 2280

6841 TCC TAC GAC CCC AAC CTG GAG GAC GGC GAC CAG ACG CCG CTC AGT TTC CAC TGG GCC TGT 6900
2281 S Y D P N L E D G D Q T P L S F H W A C 2300

6901 GTG GCT TCG ACA CAG AGG GAG GCT GGC GGG TGT GCG CTG AAC TTT GGG CCC CGC GGG AGC 6960
2301 V A S T Q R E A G G C A L N F G P R G S 2320

6961 AGC ACG GTC ACC ATT CCA CGG GAG CGG CTG GCG GCT GGC GTG GAG TAC ACC TTC AGC CTG 7020
2321 S T V T I P R E R L A A G V E Y T F S L 2340

7021 ACC GTG TGG AAG GCC GGC CGC AAG GAG GAG GCC ACC AAC CAG ACG GTG CTG ATC CGG AGT 7080
2341 T V W K A G R K E E A T N Q T V L I R S 2360

7081 GGC CGG GTG CCC ATT GTG TCC TTG GAG TGT GTG TCC TGC AAG GCA CAG GCC GTG TAC GAA 7140
2361 G R V P I V S L E C V S C K A Q A V Y E 2380

7141 GTG AGC CGC AGC TCC TAC GTG TAC TTG GAG GGC CGC TGC CTC AAT TGC AGC AGC GGC TCC 7200
2381 V S R S S Y V Y L E G R C L N C S S G S 2400

FIG.6I

7201 AAG CGA GGG CGG TGG GCT GCA CGT ACG TTC AGC AAC AAG ACG CTG GTG CTG GAT GAG ACC 7260
2401 K R G R W A A R T F S N K T L V L D E T 2420

7261 ACC ACA TCC ACG GGC AGT GCA GGC ATG CGA CTG GTG CTG CGG CGG GGC GTG CTG CGG GAC 7320
2421 T T S T G S A G M R L V L R R G V L R D 2440

7321 GGC GAG GGA TAC ACC TTC ACG CTC ACG GTG CTG GGC CGC TCT GGC GAG GAG GAG GGC TGC 7380
2441 G E G Y T F T L T V L G R S G E E E G C 2460

7381 GCC TCC ATC CGC CTG TCC CCC AAC CGC CCG CCG CTG GGG GGC TCT TGC CGC CTC TTC CCA 7440
2461 A S I R L S P N R P P L G G S C R L F P 2480

7441 CTG GGC GCT GTG CAC GCC CTC ACC ACC AAG GTG CAC TTC GAA TGC ACG GGC TGG CAT GAC 7500
2481 L G A V H A L T T K V H F E C T G W H D 2500

7501 GCG GAG GAT GCT GGC GCC CCG CTG GTG TAC GCC CTG CTG CTG CGG CGC TGT CGC CAG GGC 7560
2501 A E D A G A P L V Y A L L L R R C R Q G 2520

7561 CAC TGC GAG GAG TTC TGT GTC TAC AAG GGC AGC CTC TCC AGC TAC GGA GCC GTG CTG CCC 7620
2521 H C E E F C V Y K G S L S S Y G A V L P 2540

7621 CCG GGT TTC AGG CCA CAC TTC GAG GTG GGC CTG GCC GTG GTG GTG CAG GAC CAG CTG GGA 7680
2541 P G F R P H F E V G L A V V V Q D Q L G 2560

7681 GCC GCT GTG GTC GCC CTC AAC AGG TCT TTG GCC ATC ACC CTC CCA GAG CCC AAC GGC AGC 7740
2561 A A V V A L N R S L A I T L P E P N G S 2580

7741 GCA ACG GGG CTC ACA GTC TGG CTG CAC GGG CTC ACC GCT AGT GTG CTC CCA GGG CTG CTG 7800
2581 A T G L T V W L H G L T A S V L P G L L 2600

7801 CGG CAG GCC GAT CCC CAG CAC GTC ATC GAG TAC TCG TTG GCC CTG GTC ACC GTG CTG AAC 7860
2601 R Q A D P Q H V I E Y S L A L V T V L N 2620

7861 GAG TAC GAG CGG GCC CTG GAC GTG GCG GCA GAG CCC AAG CAC GAG CGG CAG CAC CGA GCC 7920
2621 E Y E R A L D V A A E P K H E R Q H R A 2640

7921 CAG ATA CGC AAG AAC ATC ACG GAG ACT CTG GTG TCC CTG AGG GTC CAC ACT GTG GAT GAC 7980
2641 Q I R K N I T E T L V S L R V H T V D D 2660

FIG.6J

7981 ATC CAG CAG ATC GCT GCT GCG CTG GCC CAG TGC ATG GGG CCC AGC AGG GAG CTC GTA TGC 8040
2661 I Q Q I A A A L A Q C M G P S R E L V C 2680

8041 CGC TCG TGC CTG AAG CAG ACG CTG CAC AAG CTG GAG GCC ATG ATG CTC ATC CTG CAG GCA 8100
2681 R S C L K Q T L H K L E A M M L I L Q A 2700

8101 GAG ACC ACC GCG GGC ACC GTG ACG CCC ACC GCC ATC GGA GAC AGC ATC CTC AAC ATC ACA 8160
2701 E T T A G T V T P T A I G D S I L N I T 2720

8161 GGA GAC CTC ATC CAC CTG GCC AGC TCG GAC GTG CGG GCA CCA CAG CCC TCA GAG CTG GGA 8220
2721 G D L I H L A S S D V R A P Q P S E L G 2740

8221 GCC GAG TCA CCA TCT CGG ATG GTG GCG TCC CAG GCC TAC AAC CTG ACC TCT GCC CTC ATG 8280
2741 A E S P S R M V A S Q A Y N L T S A L M 2760

8281 CGC ATC CTC ATG CGC TCC CGC GTG CTC AAC GAG GAG CCC CTG ACG CTG GCG GGC GAG GAG 8340
2761 R I L M R S R V L N E E P L T L A G E E 2780

8341 ATC GTG GCC CAG GGC AAG CGC TCG GAC CCG CGG AGC CTG CTG TGC TAT GGC GGC GCC CCA 8400
2781 I V A Q G K R S D P R S L L C Y G G A P 2800

8401 GGG CCT GGC TGC CAC TTC TCC ATC CCC GAG GCT TTC AGC GGG GCC CTG GCC AAC CTC AGT 8460
2801 G P G C H F S I P E A F S G A L A N L S 2820

8461 GAC GTG GTG CAG CTC ATC TTT CTG GTG GAC TCC AAT CCC TTT CCC TTT GGC TAT ATC AGC 8520
2821 D V V Q L I F L V D S N P F P F G Y I S 2840

8521 AAC TAC ACC GTC TCC ACC AAG GTG GCC TCG ATG GCA TTC CAG ACA CAG GCC GGC GCC CAG 8580
2841 N Y T V S T K V A S M A F Q T Q A G A Q 2860

8581 ATC CCC ATC GAG CGG CTG GCC TCA GAG CGC GCC ATC ACC GTG AAG GTG CCC AAC AAC TCG 8640
2861 I P I E R L A S E R A I T V K V P N N S 2880

8641 GAC TGG GCT GCC CGG GGC CAC CGC AGC TCC GCC AAC TCC GCC AAC TCC GTT GTG GTC CAG 8700
2881 D W A A R G H R S S A N S A N S V V V Q 2900

8701 CCC CAG GCC TCC GTC GGT GCT GTG GTC ACC CTG GAC AGC AGC AAC CCT GCG GCC GGG CTG 8760
2901 P Q A S V G A V V T L D S S N P A A G L 2920

FIG.6K

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8761  CAT CTG CAG CTC AAC TAT ACG CTG CTG GAC GGC CAC TAC CTG TCT GAG GAA CCT GAG CCC  8820
2921  H   L   Q   L   N   Y   T   L   L   D   G   H   Y   L   S   E   E   P   E   P    2940

8821  TAC CTG GCA GTC TAC CTA CAC TCG GAG CCC CGG CCC AAT GAG CAC AAC TGC TCG GCT AGC  8880
2941  Y   L   A   V   Y   L   H   S   E   P   R   P   N   E   H   N   C   S   A   S    2960

8881  AGG AGG ATC CGC CCA GAG TCA CTC CAG GGT GCT GAC CAC CGG CCC TAC ACC TTC TTC ATT  8940
2961  R   R   I   R   P   E   S   L   Q   G   A   D   H   R   P   Y   T   F   F   I    2980

8941  TCC CCG GGG AGC AGA GAC CCA GCG GGG AGT TAC CAT CTG AAC CTC TCC AGC CAC TTC CGC  9000
2981  S   P   G   S   R   D   P   A   G   S   Y   H   L   N   L   S   S   H   F   R    3000

9001  TGG TCG GCG CTG CAG GTG TCC GTG GGC CTG TAC ACG TCC CTG TGC CAG TAC TTC AGC GAG  9060
3001  W   S   A   L   Q   V   S   V   G   L   Y   T   S   L   C   Q   Y   F   S   E    3020

9061  GAG GAC ATG GTG TGG CGG ACA GAG GGG CTG CTG CCC CTG GAG GAG ACC TCG CCC CGC CAG  9120
3021  E   D   M   V   W   R   T   E   G   L   L   P   L   E   E   T   S   P   R   Q    3040

9121  GCC GTC TGC CTC ACC CGC CAC CTC ACC GCC TTC GGC GCC AGC CTC TTC GTG CCC CCA AGC  9180
3041  A   V   C   L   T   R   H   L   T   A   F   G   A   S   L   F   V   P   P   S    3060

9181  CAT GTC CGC TTT GTG TTT CCT GAG CCG ACA GCG GAT GTA AAC TAC ATC GTC ATG CTG ACA  9240
3061  H   V   R   F   V   F   P   E   P   T   A   D   V   N   Y   I   V   M   L   T    3080

9241  TGT GCT GTG TGC CTG GTG ACC TAC ATG GTC ATG GCC GCC ATC CTG CAC AAG CTG GAC CAG  9300
3081  C   A   V   C   L   V   T   Y   M   V   M   A   A   I   L   H   K   L   D   Q    3100

9301  TTG GAT GCC AGC CGG GGC CGC GCC ATC CCT TTC TGT GGG CAG CGG GGC CGC TTC AAG TAC  9360
3101  L   D   A   S   R   G   R   A   I   P   F   C   G   Q   R   G   R   F   K   Y    3120

9361  GAG ATC CTC GTC AAG ACA GGC TGG GGC CGG GGC TCA GGT ACC ACG GCC CAC GTG GGC ATC  9420
3121  E   I   L   V   K   T   G   W   G   R   G   S   G   T   T   A   H   V   G   I    3140

9421  ATG CTG TAT GGG GTG GAC AGC CGG AGC GGC CAC CGG CAC CTG GAC GGC GAC AGA GCC TTC  9480
3141  M   L   Y   G   V   D   S   R   S   G   H   R   H   L   D   G   D   R   A   F    3160

9481  CAC CGC AAC AGC CTG GAC ATC TTC CGG ATC GCC ACC CCG CAC AGC CTG GGT AGC GTG TGG  9540
3161  H   R   N   S   L   D   I   F   R   I   A   T   P   H   S   L   G   S   V   W    3180

9541  AAG ATC CGA GTG TGG CAC GAC AAC AAA GGG CTC AGC CCT GCC TGG TTC CTG CAG CAC GTC  9600
3181  K   I   R   V   W   H   D   N   K   G   L   S   P   A   W   F   L   Q   H   V    3200
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FIG.6L

9601 ATC GTC AGG GAC CTG CAG ACG GCA CGC AGC GCC TTC TTC CTG GTC AAT GAC TGG CTT TCG 9660
3201 I V R D L Q T A R S A F F L V N D W L S 3220

9661 GTG GAG ACG GAG GCC AAC GGG GGC CTG GTG GAG AAG GAG GTG CTG GCC GCG AGC GAC GCA 9720
3221 V E T E A N G G L V E K E V L A A S D A 3240

9721 GCC CTT TTG CGC TTC CGG CGC CTG CTG GTG GCT GAG CTG CAG CGT GGC TTC TTT GAC AAG 9780
3241 A L L R F R R L L V A E L Q R G F F D K 3260

9781 CAC ATC TGG CTC TCC ATA TGG GAC CGG CCG CCT CGT AGC CGT TTC ACT CGC ATC CAG AGG 9840
3261 H I W L S I W D R P P R S R F T R I Q R 3280

9841 GCC ACC TGC TGC GTT CTC CTC ATC TGC CTC TTC CTG GGC GCC AAC GCC GTG TGG TAC GGG 9900
3281 A T C C V L L I C L F L G A N A V W Y G 3300

9901 GCT GTT GGC GAC TCT GCC TAC AGC ACG GGG CAT GTG TCC AGG CTG AGC CCG CTG AGC GTC 9960
3301 A V G D S A Y S T G H V S R L S P L S V 3320

9961 GAC ACA GTC GCT GTT GGC CTG GTG TCC AGC GTG GTT GTC TAT CCC GTC TAC CTG GCC ATC 10020
3321 D T V A V G L V S S V V V Y P V Y L A I 3340

10021 CTT TTT CTC TTC CGG ATG TCC CGG AGC AAG GTG GCT GGG AGC CCG AGC CCC ACA CCT GCC 10080
3341 L F L F R M S R S K V A G S P S P T P A 3360

10081 GGG CAG CAG GTG CTG GAC ATC GAC AGC TGC CTG GAC TCG TCC GTG CTG GAC AGC TCC TTC 10140
3361 G Q Q V L D I D S C L D S S V L D S S F 3380

10141 CTC ACG TTC TCA GGC CTC CAC GCT GAG CAG GCC TTT GTT GGA CAG ATG AAG AGT GAC TTG 10200
3381 L T F S G L H A E Q A F V G Q M K S D L 3400

10201 TTT CTG GAT GAT TCT AAG AGT CTG GTG TGC TGG CCC TCC GGC GAG GGA ACG CTC AGT TGG 10260
3401 F L D D S K S L V C W P S G E G T L S W 3420

10261 CCG GAC CTG CTC AGT GAC CCG TCC ATT GTG GGT AGC AAT CTG CGG CAG CTG GCA CGG GGC 10320
3421 P D L L S D P S I V G S N L R Q L A R G 3440

10321 CAG GCG GGC CAT GGG CTG GGC CCA GAG GAG GAC GGC TTC TCC CTG GCC AGC CCC TAC TCG 10380
3441 Q A G H G L G P E E D G F S L A S P Y S 3460

10381 CCT GCC AAA TCC TTC TCA GCA TCA GAT GAA GAC CTG ATC CAG CAG GTC CTT GCC GAG GGG 10440
3461 P A K S F S A S D E D L I Q Q V L A E G 3480

FIG.6M

10441 GTC AGC AGC CCA GCC CCT ACC CAA GAC ACC CAC ATG GAA ACG GAC CTG CTC AGC AGC CTG 10500
3481 V S S P A P T Q D T H M E T D L L S S L 3500

10501 TCC AGC ACT CCT GGG GAG AAG ACA GAG ACG CTG GCG CTG CAG AGG CTG GGG GAG CTG GGG 10560
3501 S S T P G E K T E T L A L Q R L G E L G 3520

10561 CCA CCC AGC CCA GGC CTG AAC TGG GAA CAG CCC CAG GCA GCG AGG CTG TCC AGG ACA GGA 10620
3521 P P S P G L N W E Q P Q A A R L S R T G 3540

10621 CTG GTG GAG GGT CTG CGG AAG CGC CTG CTG CCG GCC TGG TGT GCC TCC CTG GCC CAC GGG 10680
3541 L V E G L R K R L L P A W C A S L A H G 3560

10681 CTC AGC CTG CTC CTG GTG GCT GTG GCT GTG GCT GTC TCA GGG TGG GTG GGT GCG AGC TTC 10740
3561 L S L L L V A V A V A V S G W V G A S F 3580

10741 CCC CCG GGC GTG AGT GTT GCG TGG CTC CTG TCC AGC AGC GCC AGC TTC CTG GCC TCA TTC 10800
3581 P P G V S V A W L L S S S A S F L A S F 3600

10801 CTC GGC TGG GAG CCA CTG AAG GTC TTG CTG GAA GCC CTG TAC TTC TCA CTG GTG GCC AAG 10860
3601 L G W E P L K V L L E A L Y F S L V A K 3620

10861 CGG CTG CAC CCG GAT GAA GAT GAC ACC CTG GTA GAG AGC CCG GCT GTG ACG CCT GTG AGC 10920
3621 R L H P D E D D T L V E S P A V T P V S 3640

10921 GCA CGT GTG CCC CGC GTA CGG CCA CCC CAC GGC TTT GCA CTC TTC CTG GCC AAG GAA GAA 10980
3641 A R V P R V R P P H G F A L F L A K E E 3660

10981 GCC CGC AAG GTC AAG AGG CTA CAT GGC ATG CTG CGG AGC CTC CTG GTG TAC ATG CTT TTT 11040
3661 A R K V K R L H G M L R S L L V Y M L F 3680

11041 CTG CTG GTG ACC CTG CTG GCC AGC TAT GGG GAT GCC TCA TGC CAT GGG CAC GCC TAC CGT 11100
3681 L L V T L L A S Y G D A S C H G H A Y R 3700

11101 CTG CAA AGC GCC ATC AAG CAG GAG CTG CAC AGC CGG GCC TTC CTG GCC ATC ACG CGG TCT 11160
3701 L Q S A I K Q E L H S R A F L A I T R S 3720

11161 GAG GAG CTC TGG CCA TGG ATG GCC CAC GTG CTG CTG CCC TAC GTC CAC GGG AAC CAG TCC 11220
3721 E E L W P W M A H V L L P Y V H G N Q S 3740

11221 AGC CCA GAG CTG GGG CCC CCA CGG CTG CGG CAG GTG CGG CTG CAG GAA GCA CTC TAC CCA 11280
3741 S P E L G P P R L R Q V R L Q E A L Y P 3760

FIG.6N

11281 GAC CCT CCC GGC CCC AGG GTC CAC ACG TGC TCG GCC GCA GGA GGC TTC AGC ACC AGC GAT 11340
3761 D P P G P R V H T C S A A G G F S T S D 3780

11341 TAC GAC GTT GGC TGG GAG AGT CCT CAC AAT GGC TCG GGG ACG TGG GCC TAT TCA GCG CCG 11400
3781 Y D V G W E S P H N G S G T W A Y S A P 3800

11401 GAT CTG CTG GGG GCA TGG TCC TGG GGC TCC TGT GCC GTG TAT GAC AGC GGG GGC TAC GTG 11460
3801 D L L G A W S W G S C A V Y D S G G Y V 3820

11461 CAG GAG CTG GGC CTG AGC CTG GAG GAG AGC CGC GAC CGG CTG CGC TTC CTG CAG CTG CAC 11520
3821 Q E L G L S L E E S R D R L R F L Q L H 3840

11521 AAC TGG CTG GAC AAC AGG AGC CGC GCT GTG TTC CTG GAG CTC ACG CGC TAC AGC CCG GCC 11580
3841 N W L D N R S R A V F L E L T R Y S P A 3860

11581 GTG GGG CTG CAC GCC GCC GTC ACG CTG CGC CTC GAG TTC CCG GCG GCC GGC CGC GCC CTG 11640
3861 V G L H A A V T L R L E F P A A G R A L 3880

11641 GCC GCC CTC AGC GTC CGC CCC TTT GCG CTG CGC CGC CTC AGC GCG GGC CTC TCG CTG CCT 11700
3881 A A L S V R P F A L R R L S A G L S L P 3900

11701 CTG CTC ACC TCG GTG TGC CTG CTG CTG TTC GCC GTG CAC TTC GCC GTG GCC GAG GCC CGT 11760
3901 L L T S V C L L L F A V H F A V A E A R 3920

11761 ACT TGG CAC AGG GAA GGG CGC TGG CGC GTG CTG CGG CTC GGA GCC TGG GCG CGG TGG CTG 11820
3921 T W H R E G R W R V L R L G A W A R W L 3940

11821 CTG GTG GCG CTG ACG GCG GCC ACG GCA CTG GTA CGC CTC GCC CAG CTG GGT GCC GCT GAC 11880
3941 L V A L T A A T A L V R L A Q L G A A D 3960

11881 CGC CAG TGG ACC CGT TTC GTG CGC GGC CGC CCG CGC CGC TTC ACT AGC TTC GAC CAG GTG 11940
3961 R Q W T R F V R G R P R R F T S F D Q V 3980

11941 GCG CAC GTG AGC TCC GCA GCC CGT GGC CTG GCG GCC TCG CTG CTC TTC CTG CTT TTG GTC 12000
3981 A H V S S A A R G L A A S L L F L L L V 4000

12001 AAG GCT GCC CAG CAC GTA CGC TTC GTG CGC CAG TGG TCC GTC TTT GGC AAG ACA TTA TGC 12060
4001 K A A Q H V R F V R Q W S V F G K T L C 4020

12061 CGA GCT CTG CCA GAG CTC CTG GGG GTC ACC TTG GGC CTG GTG GTG CTC GGG GTA GCC TAC 12120
4021 R A L P E L L G V T L G L V V L G V A Y 4040

FIG.60

12121 GCC CAG CTG GCC ATC CTG CTC GTG TCT TCC TGT GTG GAC TCC CTC TGG AGC GTG GCC CAG 12180
4041 A Q L A I L L V S S C V D S L W S V A Q 4060

12181 GCC CTG TTG GTG CTG TGC CCT GGG ACT GGG CTC TCT ACC CTG TGT CCT GCC GAG TCC TGG 12240
4061 A L L V L C P G T G L S T L C P A E S W 4080

12241 CAC CTG TCA CCC CTG CTG TGT GTG GGG CTC TGG GCA CTG CGG CTG TGG GGC GCC CTA CGG 12300
4081 H L S P L L C V G L W A L R L W G A L R 4100

12301 CTG GGG GCT GTT ATT CTC CGC TGG CGC TAC CAC GCC TTG CGT GGA GAG CTG TAC CGG CCG 12360
4101 L G A V I L R W R Y H A L R G E L Y R P 4120

12361 GCC TGG GAG CCC CAG GAC TAC GAG ATG GTG GAG TTG TTC CTG CGC AGG CTG CGC CTC TGG 12420
4121 A W E P Q D Y E M V E L F L R R L R L W 4140

12421 ATG GGC CTC AGC AAG GTC AAG GAG TTC CGC CAC AAA GTC CGC TTT GAA GGG ATG GAG CCG 12480
4141 M G L S K V K E F R H K V R F E G M E P 4160

12481 CTG CCC TCT CGC TCC TCC AGG GGC TCC AAG GTA TCC CCG GAT GTG CCC CCA CCC AGC GCT 12540
4161 L P S R S S R G S K V S P D V P P P S A 4180

12541 GGC TCC GAT GCC TCG CAC CCC TCC ACC TCC TCC AGC CAG CTG GAT GGG CTG AGC GTG AGC 12600
4181 G S D A S H P S T S S S Q L D G L S V S 4200

12601 CTG GGC CGG CTG GGG ACA AGG TGT GAG CCT GAG CCC TCC CGC CTC CAA GCC GTG TTC GAG 12660
4201 L G R L G T R C E P E P S R L Q A V F E 4220

12661 GCC CTG CTC ACC CAG TTT GAC CGA CTC AAC CAG GCC ACA GAG GAC GTC TAC CAG CTG GAG 12720
4221 A L L T Q F D R L N Q A T E D V Y Q L E 4240

12721 CAG CAG CTG CAC AGC CTG CAA GGC CGC AGG AGC AGC CGG GCG CCC GCC GGA TCT TCC CGT 12780
4241 Q Q L H S L Q G R R S S R A P A G S S R 4260

12781 GGC CCA TCC CCG GGC CTG CGG CCA GCA CTG CCC AGC CGC CTT GCC CGG GCC AGT CGG GGT 12840
4261 G P S P G L R P A L P S R L A R A S R G 4280

12841 GTG GAC CTG GCC ACT GGC CCC AGC AGG ACA CCC CTT CGG GCC AAG AAC AAG GTC CAC CCC 12900
4281 V D L A T G P S R T P L R A K N K V H P 4300

12901 AGC AGC ACT TAG
4301 S S T *

FIG.6P

SUBSTITUTE SHEET (RULE 26)

1 MPPAAPARLA LALGLGLWLG ALAGGPGRGC GPCEPPCLCG PAPGAACRVN CSGRGLRTLG PALRIPADAT ELDVSHNLLR 80

SIGNAL PEPTIDE LRR CYTEINE-RICH AMINO TERMNUS

81 ALDVGLLANL SALAELDISN NKISTLEEGI FANLFNLSEI NLSGNPFECD-CGLAWLPQWA EEQQVRVVQP EAATCAGPGS 160

LRR1 LRR2 LRR CYTEINE-RICH CARBOXY TERMNUS

161 LAGQPLLGIP LLDSGCGEEY VACLPDNSSG TVAAVSFSAA HEGLLQPEAC SAFCCSTGQG LAALSEQGWC LCGAAQPSSA 240

241 SFACLSLCSG PPAPPAPTCR GPTLLQHVFP ASPGATLVGP HGPLASGQLA AFHIAAPLPV TDTRWDFGDG SAEVDAAGPA 320

PKD1 R1

321 ASHRYVLPGR YHVTAVLALG AGSALLGTDV QVEAAPAALE LVCPSSVQSD ESLDLSIQNR GGSGLEAAYS IVALGEEPAR 400

401 AVHPLCPSDT EIFPGNGHCY RLVVEKAAWL QAQEQCQAWA GAALAMVDSP AVQRFLVSRV TRSLDVWIGF STVQGVEVGP 480

C-TYPE LECTIN BINDING DOMAIN

481 APQGEAFSLE SCQNWLPGEP HPATAEHCVR LGPTGWCNTD LCSAPHSYVC ELQPGGPVQD AENLLVGAPS GDLQGPLTPL 560

561 AQQDGLSAPH EPVEVMVFPG LRLSREAFLT TAEFGTQELR RPAQLRLQVY RLLSTAGTPE NGSEPESRSP DNRTQLAPAC 640

641 MPGGRWCPGA NICLPLDASC HPQACANGCT SGPGLPGAPY ALWREFLFSV PAGPPAQYSV TLHGQDVLML PGDLVGLQHD 720

LDL-A

721 AGPGALLHCS PAPGHPGPRA PYLSANASSW LPHLPAQLEG TWGCPACALR LLAQREQLTV LLGLRPNPGL RLPGRYEVRA 800

801 EVGNGVSRHN LSCSFDVVSP VAGLRVIYPA PRDGRLYVPT NGSALVLQVD SGANATATAR WPGGSLSARF ENVCPALVAT 880

881 FVPACPWETN DTLFSVVALP WLSEGEHVVD VVVENSASRA NLSLRVTAEE PICGLRATPS PEARVLQGVL VRYSPVVEAG 960

961 SDMVFRWTIN DKQSLTFQNV VFNVIYQSAA VFKLSLTASN HVSNVTVNYN VTVERMNRMQ GLQVSTVPAV LSPNATLALT 1040

1041 AGVLVDSAVE VAFLWTFGDG EQALHQFQPP YNESFPVPDP SVAQVLVEHN VTHTYAAPGE YLLTVLASNA FENLTQQVPV 1120

1121 SVRASLPSVA VGVSDGVLVA GRPVTFYPHP LPSPGGVLYT WDFGDGSPVL TQSQPAANHT YASRGTYHVR LEVNNTVSGA 1200

PKD1 R3

1201 AAQADVRVFE ELRGLSVDMS LAVEQGAPVV VSAAVQTGDN ITWTFDMGDG TVLSGPEATV EHVYLRAQNC TVTVGAGSPA 1280

PKD1 R4

1281 GHLARSLHVL VFVLEVLRVE PAACIPTQPD ARLTAYVTGN PAHYLFDWTF GDGSSNTTVR GCPTVTHNFT RSGTFPLALV 1360

PKD1 R5

1361 LSSRVNRAHY FTSICVEPEV GNVTLQPERQ FVQLGDEAWL VACAWPPFPY RYTWDFGTEE AAPTRARGPE VTFIYRDPGS 1440

PKD1 R6

1441 YLVTVTASNN ISAANDSALV EVQEPVLVTS IKVNGSLGLE LQQPYLFSAV GRGRPASYLW DLGDGGWLEG PEVTHAYNST 1520

PKD1 R7

1521 GDFTVRVAGW NEVSRSEAWL NVTVKRRVRG LVVNASRTVV PLNGSVSFST SLEAGSDVRY SWVLCDRCTP IPGGPTISYT 1600

PKD1 R8

1601 FRSVGTFNII VTAENEVGSA QDSIFVYVLQ LIEGLQVVGG GRYFPTNHTV QLQAVVRDGT NVSYSWTAWR DRGPALAGSG 1680

PKD1 R9

1681 KGFSLTVLEA GTYHVQLRAT NMLGSAWADC TMDFVEPVGW LMVAASPNPA AVNTSVTLSA ELAGGSGVVY TWSLEEGLSW 1760

PKD1 R10

1761 ETSEPFTTHS FPTPGLHLVT MTAGNPLGSA NATVEVDVQV PVSGLSIRAS EPGGSFVAAG SSVPFWGQLA TGTNVSWCWA 1840

PKD1 R11

FIG.7A

1841 VPGGSSKRGP HVTMVFPDAG TFSIRLNASN AVSWVSATYN LTAEEPIVGL VLWASSKVVA PGQLVHFQIL LAAGSAVTFR 1920
PKD1 R12
1921 LQVGGANPEV LPGPRFSHSF PRVGDHVVSV RGKNHVSWAQ AQVRIVVLEA VSGLQVPNCC EPGIATGTER NFTARVQRGS 2000
PKD1 R13
2001 RVAYAWYFSL QKVQGDSLVI LSGRDVTYTP VAAGLLEIQV RAFNALGSEN RTLVLEVQDA VQYVALQSGP CFTNRSAQFE 2080
2081 AATSPSPRRV AYHWDFGDGS PGQDTDEPRA EHSYLRPGDY RVQVNASNLV SFFVAQATVT VQVLACREPE VDVVLPLQVL 2160
PKD1 R14
2161 MRRSQRNYLE AHVDLRDCVT YQTEYRWEVY RTASCQRPGR PARVALPGVD VSRPRLVLPR LALPVGHYCF VFVVSFGDTP 2240
2241 LTQSIQANVT VAPERLVPII EGGSYRVWSD TRDLVLDGSE SYDPNLEDGD QTPLSFHWAC VASTQREAGG CALNFGPRGS 2320
2321 STVTIPRERL AAGVEYTFSL TVWKAGRKEE ATNQTVLIRS GRVPIVSLEC VSCKAQAVYE VSRSSYVYLE GRCLNCSSGS 2400
2401 KRGRWAARTF SNKTLVLDET TTSTGSAGMR LVLRRGVLRD GEGYTFTLTV LGRSGEEEGC ASIRLSPNRP PLGGSCRLFP 2480
2481 LGAVHALTTK VHFECTGWHD AEDAGAPLVY ALLLRRCRQG HCEEFCVYKG SLSSYGAVLP PGFRPHFEVG LAVVVQDQLG 2560
2561 AAVVALNRSL AITLPEPNGS ATGLTVWLHG LTASVLPGLL RQADPQHVIE YSLALVTVLN EYERALDVAA EPKHERQHRA 2640
2641 QIRKNITETL VSLRVHTVDD IQQIAAALAQ CMGPSRELVC RSCLKQTLHK LEAMMLILQA ETTAGTVTPT AIGDSILNIT 2720
2721 GDLIHLASSD VRAPQPSELG AESPSRMVAS QAYNLTSALM RILMRSRVLN EEPLTLAGEE IVAQGKRSDP RSLLCYGGAP 2800
2801 GPGCHFSIPE AFSGALANLS DVVQLIFLVD SNPFPFGYIS NYTVSTKVAS MAFQTQAGAQ IPIERLASER AITVKVPNNS 2880
2881 DWAARGHRSS ANSANSVVVQ PQASVGAVVT LDSSNPAAGL HLQLNYTLLD GHYLSEEPEP YLAVYLHSEP RPNEHNCSAS 2960
2961 RRIRPESLQG ADHRPYTFFI SPGSRDPAGS YHLNLSSHFR WSALQVSVGL YTSLCQYFSE EDMVWRTEGL LPLEETSPRQ 3040
3041 AVCLTRHLTA FGASLFVPPS HVRFVFPEPT ADVNYIVMLT CAVCLVTYMV MAAILHKLDQ LDASRGRAIP FCGQRGRFKY 3120
3121 EILVKTGWGR GSGTTAHVGI MLYGVDSRSG HRHLDGDRAF HRNSLDIFRI ATPHSLGSVW KIRVWHDNKG LSPAWFLQHV 3200
3201 IVRDLQTARS AFFLVNDWLS VETEANGGLV EKEVLAASDA ALLRFRRLLV AELQRGFFDK HIWLSIWDRP PRSRFTRIQR 3280
3281 ATCCVLLICL FLGANAVWYG AVGDSAYSTG HVSRLSPLSV DTVAVGLVSS VVVYPVYLAI LFLFRMSRSK VAGSPSPTPA 3360
3361 GQQVLDIDSC LDSSVLDSSF LTFSGLHAEQ AFVGQMKSDL FLDDSKSLVC WPSGEGTLSW PDLLSDPSIV GSNLRQLARG 3440
3441 QAGHGLGPEE DGFSLASPYS PAKSFSASDE DLIQQVLAEG VSSPAPTQDT HMETDLLSSL SSTPGEKTET LALQRLGELG 3520
3521 PPSPGLNWEQ PQAARLSRTG LVEGLRKRLL PAWCASLAHG LSLLLVAVAV AVSGWVGASF PPGVSVAWLL SSSASFLASF 3600
3601 LGWEPLKVLL EALYFSLVAK RLHPDEDDTL VESPAVTPVS ARVPRVRPPH GFALFLAKEE ARKVKRLHGM LRSLLVYMLF 3680
3681 LLVTLLASYG DASCHGHAYR LQSAIKQELH SRAFLAITRS EELWPWMAHV LLPYVHGNQS SPELGPPRLR QVRLQEALYP 3760
3761 DPPGPRVHTC SAAGGFSTSD YDVGWESPHN GSGTWAYSAP DLLGAWSWGS CAVYDSGGYV QELGLSLEES RDRLRFLQLH 3840
3841 NWLDNRSRAV FLELTRYSPA VGLHAAVTLR LEFPAAGRAL AALSVRPFAL RRLSAGLSLP LLTSVCLLLF AVHFAVAEAR 3920
3921 TWHREGRWRV LRLGAWARWL LVALTAATAL VRLAQLGAAD RQWTRFVRGR PRRFTSFDQV AHVSSAARGL AASLLFLLLV 4000
4001 KAAQHVRFVR QWSVFGKTLC RALPELLGVT LGLVVLGVAY AQLAILLVSS CVDSLWSVAQ ALLVLCPGTG LSTLCPAESW 4080
4081 HLSPLLCVGL WALRLWGALR LGAVILRWRY HALRGELYRP AWEPQDYEMV ELFLRRLRLW MGLSKVKEFR HKVRFEGMEP 4160
4161 LPSRSSRGSK VSPDVPPPSA GSDASHPSTS SSQLDGLSVS LGRLGTRCEP EPSRLQAVFE ALLTQFDRLN QATEDVYQLE 4240
4241 QQLHSLQGRR SSRAPAGSSR GPSPGLRPAL PSRLARASRG VDLATGPSRT PLRAKNKVHP SSTZ 4304

FIG.7B

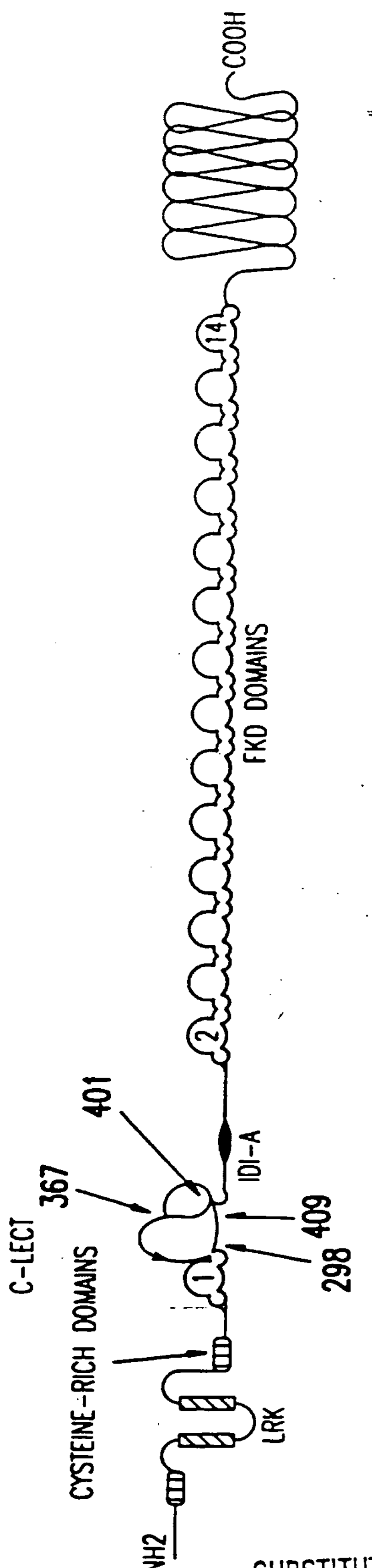
COOH
14
FKD DOMAINS
2
401
IDI-A
367
409
C-LECT
298
1
CYSTEINE-RICH DOMAINS
LRK
NH2

FIG.8

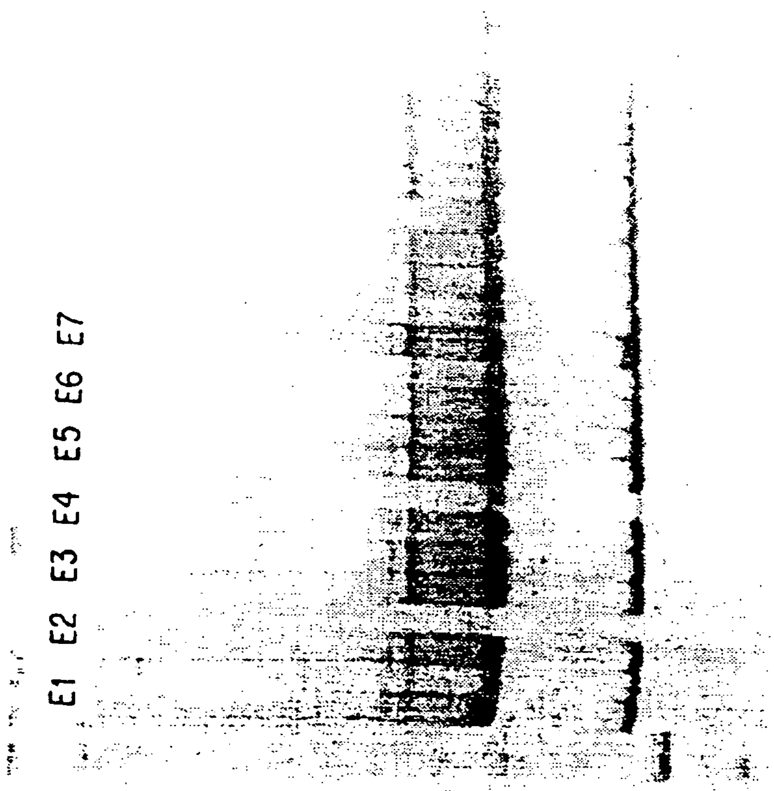

FIG.9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07079

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :435/6, 69.1, 172.3, 240.1, 243, 320.1; 536/23.5, 24.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.1, 172.3, 240.1, 243, 320.1; 536/23.5, 24.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GENOMICS, Volume 18, issued 1993, Kimberling et al., "Autosomal Dominant Polycystic Kidney Disease: Localization of the Second Gene to Chromosome 4q13-q23", pages 467-472, see entire document.	1-6, 10-13, and 16
Y	HUMAN GENETICS, Volume 90, issued 1993, Wright et al., "A study of genetic linkage heterogeneity in 35 adult-onset polycystic kidney disease families", pages 569-571, see entire document.	1-6, 10-13, and 16
Y	GENOMICS, Volume 13, issued 1992, Himmelbauer et al., "Human-Mouse Homologies in the Region of the Polycystic Kidney Disease Gene (PKD1)", pages 35-38, see entire document.	1-6, 10-13, and 16

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

30 AUGUST 1995

Date of mailing of the international search report

25 SEP 1995

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CLINICAL CHEMISTRY, Volume 35, issued 1989, Reeders et al., "Mapping the Locus of Autosomal Dominant Polycystic Kidney Disease: Diagnostic Application", pages B13-16, see entire document.	1-6, 10-13, and 16
Y	JOURNAL OF MEDICAL GENETICS, Volume 27, issued 1990, Breuning et al., "Mapping of 16 polymorphic loci on the short arm of chromosome 16 close to the polycystic kidney disease gene (PKD1)", pages 603-613, see entire document.	1-6, 10-13, and 16
Y	GENOMICS, Volume 13, issued 1992, Germino et al., "The Gene for Autosomal Dominant Polycystic Kidney Disease Lies in a 750-kb CpG-Rich Region", pages 144-151, see entire document.	1-6, 10-13, and 16
Y	AMERICAN JOURNAL OF HUMAN GENETICS, Volume 46, issued 1990, Germino et al., "Identification of a Locus Which Shows No Genetic Recombination with the Autosomal Dominant Polycystic Kidney Disease Gene on Chromosome 16", pages 925-933, see entire document.	1-6, 10-13, and 16
Y	NUCLEIC ACIDS RESEARCH, Volume 18, Number 23, issued 1990, Gillespie et al., "Cosmid walking and chromosome jumping in the region of PKD1 reveal a locus duplication and three CpG islands", pages 7071-7075, see entire document.	1-6, 10-13, and 16
Y	NATURE GENETICS, Volume 1, issued July 1992, Reeders, "Multilocus polycystic disease", pages 235-237, see entire document.	1-6, 10-13, and 16
Y	GENOMICS, Volume 13, issued 1992, Smolo et al., "Fine Genetic Localization of the Gene for Autosomal Dominant Polycystic Kidney Disease (PKD1) with Respect to Physically Mapped Markers", pages 152-158, see entire document.	1-6, 10-13, and 16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07079

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-6, 10-13, and 16

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07079

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07H 21/02, 21/04; C12N 1/00, 5/00, 15/00, 15/09, 15/10, 15/11, 15/63, 15/70, 15/74, 15/79

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Databases Search: APS, CAS, CA, EMBASE, BIOSIS, MEDLINE, GENBANK, ENTREE
Search terms: kidney#, polycyst?; cyst?; apkd?; pkd?; gg?; autosom?; dominant; chromosome; 16; adult;

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

This application also contains claims directed to more than one species of the generic invention as set forth below in each group. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be examined, the appropriate additional examination fees must be paid. The Groups and species are as follows:

Group I, claim(s) 1-6, 10, 11-13, and 16, drawn to a first set of products which are the DNA encoding a polycystic kidney disease-1 (PKD1) gene product, vectors containing the DNA, and host cells containing the vectors and a method of use of the DNA which is a process of diagnosing a mutant PKD1 gene.

The claims are deemed to correspond to the species listed below in the following manner: The method of use claim 16 contains two (2) species, the first listed of which is detection of the gene (which is for example classified and searched in Class 435, subclass 6) and the second of which is detection of the gene product (which is for example classified and searched in Class 435, subclass 7.1).Group II, claim(s) 7-9, 14, 15, 17-23, and 33-35, drawn to a process of treating PKD1 disease via application of compounds that inhibit expression or activity of a mutant PKD1 gene product wherein the claims corresponding to the species are listed below. The first species is an antisense molecule (claims 7, 8, and 18 which are for example classified in Class 514, subclass 44 as well as Class 435 subclass 91.31) which will be examined as the first claimed species for process claim 17. The second species is a triple helix molecule (claims 9 and 19 as for example classified in Class 514, subclass 44). The third species is a gene encoding PKD1 for replacement therapy (claims 20, 22, and 35 as for example classified in Class 514, subclass 44). The fourth species is an antibody for immunotherapy (claims 15 and 21 as for example classified in Class 424, subclass 130.1). The fifth species is the process where the PKD1 protein itself is administered (claims 14, 23, 33, and 34 as for example classified in Class 514, subclass 12).

Group III, claim(s) 24-32, drawn to a third process, which process is directed to detection of the polycystic kidney disease-1 (PKD1) gene product and monitoring of same by reaction with an antibody using a test kit is for example classified in Class 435, subclass 7.1
The inventions listed as Groups I, II, and III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: In Group I, the method of diagnosis as defined by claim 16 does not require nor result in a process of Group II, which is a method of treatment. Treatment for a condition occurs post diagnosis and does not require per se diagnosis for its practice nor does the method of diagnosis require the special technical feature(s) of a method of treatment. Insofar as Groups I and III are directed to methods of diagnosis, the method of diagnosis in Group III uses a product (an antibody) which is not the first claimed product as set forth in Group I above. Moreover, the special technical feature of the antibody is not found in the DNA, vector or host cell for which the DNA encodes the PKD1 protein. Thus, Groups I, II, and III have different special technical features that are not mutually inclusive of any one other group.

The species indicated in Groups I and II above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons:

In Group I, the second of which is detection of the gene product which is for example classified and searched in Class 435, subclass 7.1 does not require nor use the DNA used in the first listed species in claim 16. Insofar as there is a differenceinthe detected material, a protein as opposed to DNA, a protein does not have the same physical, chemical,

and biological characteristics and functionsof a DNA. In Group II, there are five species set forth in the groupings, the first of which is an antisense molecule (i.e., an oligonucleotide) which does not share the same physical, chemical, and biological properties and functions of a triple helical molecule or the gene encoding PKD1 since the gene is not an antisense molecule. Similarly, the antisense molecule is not an antibody nor is it the PKD1 protein. Here, the triple helical molecule is not found in nor is it part of any gene nor antibody nor is it the protein PKD1. Furthermore, the antibody and PKD1 are not the same protein nor does one or the other substitute for the other. Thus, where the processes recite administration of different compounds to effect a treatment, the methods of treatment have different special technical features that are not mutually inclusive, one to the other.